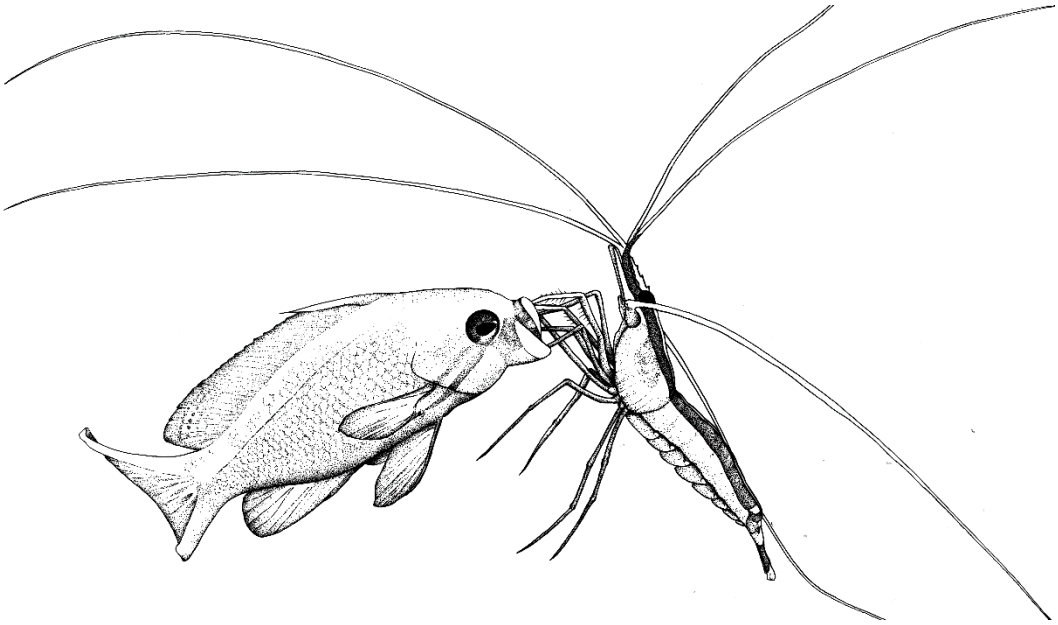
Cleaner shrimp as biocontrols in aquaculture



Thesis submitted by

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In fulfilment of the requirements for Doctorate of Philosophy (Science)

College of Science and Engineering

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Original illustration of Pseudanthias squamipinnis *being cleaned by* Lysmata amboinensis *by D. B. Vaughan, pen-and-ink*

Scholarship during candidature

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1. **Vaughan, D.B.,** Grutter, A.S., and Hutson, K.S. (2018, *in press*). Cleaner shrimp are a sustainable option to treat parasitic disease in farmed fish. *Scientific Reports* [IF = 4.122].
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4. Trujillo-González, A., Becker, J., **Vaughan, D.B.,** and Hutson, K.S. (2018). Monogenean parasites infect ornamental fish imported to Australia. *Parasitology Research*, DOI: 10.1007/s00436-018-5776-z [IF = 2.329].
5. Hutson, K.S., Brazenor, A.K., **Vaughan, D.B.,** and Trujillo-González, A. (2018). Monogenean parasite cultures: current techniques and recent advances. *Advances in Parasitology* **99**, 61–91 [IF 4.255].
6. **Vaughan, D.B.,** and Christison, K.W. (2017). *Anoplodiscus* Sonsino, 1890: a new Australian species, and the first African record from South African hosts. *Systematic Parasitology* **94**, 891–906 [IF 1.316].
7. Bastos Gomes, G., Miller, T.L., **Vaughan, D.B.,** Jerry, D.R., McCowan, C., Bradley, T., and Hutson, K.S. (2017). Evidence of multiple species of *Chilodonella* (Protozoa, Ciliophora) infecting Australian farmed freshwater fishes. *Veterinary Parasitology* **237**, 8–16 [IF. 2.278].

8. Penning, M.R., **Vaughan, D.B.,** Fivaz, K., and McEwan, T. (2017). Chapter 32. *Chemical immobilization of elasmobranchs at uShaka Sea World in Durban, South Africa*. In: M. Smith, D. Warmolts, D. Thoney, R. Hueter, M. Murray, and J. Ezcurra (Eds). The Elasmobranch Husbandry Manual 2: Recent Advances in the Care of Sharks, Rays and their Relatives, Ohio Biological Survey, USA, viii + 504 p.
9. **Vaughan, D.B.,** Grutter, A.S., Costello, M.J., and Hutson, K.S. (2016). Cleaner fishes and shrimp diversity and a re-evaluation of cleaning symbioses. *Fish and Fisheries*, DOI: 10.1111/faf.12198 [IF 9.013].
10. **Vaughan, D.B.,** Chisholm, L.A., and Hansen, H. (2016). *Electrocotyle whittingtoni* n. gen., n. sp. (Monogenea: Monocotylidae: Heterocotylinae) from the gills of a captive onefin electric ray, *Narke capensis* (Narkidae) at Two Oceans Aquarium, Cape Town, South Africa. *Parasitology Research* **115**, 3575–3584 [IF = 2.329].

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3. Mitchell, D., Christison K.W., van As, L., and **Vaughan, D.B.** (2016). Hexabothriid parasites from Rajidae species of South Africa. 45th Parasitological Society of Southern Africa Conference, South Africa. 28–30 August, 2016.
4. **Vaughan, D.B.**, Trujillo-González, A., and Hutson, K.S. (2016). Cleaner shrimp: humble janitors, double agents or skilled surgeons? 3rd International Workshop on Symbiotic Copepoda, Heron Island, Australia. 10–16 July, 2016.

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6. **Vaughan, D.B.,** and Christison, K.W. (2015). An investigation of *Amyloodinium ocellatum* in captive fish populations in South Africa and its potential impacts for finfish farming. 3rd Fisheries Research and Development Corporation Australasian Aquatic Animal Health Scientific Conference. Cairns, Australia. 6–10 July, 2015.

Statement on the contribution of others

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Chapter 2; contributors: Alexandra S. Grutter, Mark J. Costello, Kate S. Hutson.

Alexandra Grutter assisted with the conceptual idea and final editing of the associated manuscript. Mark Costello created the original version of Figure 2.2, suggested the inclusion of Figures 2.4 and 2.5, and assisted with final editing. Kate Hutson assisted with the conceptual idea, suggested the inclusion of Figure 2.1, provided the financial assistance, and provided editorial support. Citation: Vaughan, D.B., Grutter, A.S., Costello, M.J., and Hutson, K.S. (2016). Cleaner fishes and shrimp diversity and a re-evaluation of cleaning symbioses. *Fish and Fisheries*, DOI:10.1111/faf.12198.

Chapter 3; contributors: Alexandra S. Grutter, Hugh W. Ferguson, Rhondda Jones, Kate S. Hutson.

Alexandra Grutter assisted with the conceptual idea and final editing of the associated manuscript. Hugh Ferguson provided pathological interpretation of duplicate histological

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Appendix 6; contributors: Alexandra S. Grutter, Dianne Bray, Karen Cheney, Justin Marshall, and Kate S. Hutson.

Alexandra Grutter assisted with the conceptual design, and provided editorial support. Kate Hutson and Dianne Bray assisted with the conceptual idea and experimental design. Kate

Hutson provided the financial assistance for the purchase of hardware and livestock, and provided editorial support. Permission to use **Fig. A6.1** (XNite UV filter spectral analysis) was granted by Dan Llewellyn, LDP LLC www.MaxMax.com, who also supplied the image electronically. Permission to use **Fig. A6.3** (unpublished *Stenopus hispidus* spectral reflectance) was granted by Karen Cheney and Justin Marshall, who also supplied the image electronically.

Copyright note

Every reasonable effort has been made to gain permission and to acknowledge the owners of copyright material. I would be pleased to hear from any copyright owner who has been omitted or incorrectly acknowledged.

Statement on animal ethics and welfare

One thousand, four hundred and thirty six individual animals, representing 400 *Epinephelus coioides*, 520 *E. lanceolatus*, 150 *Lates calcarifer*, 126 female *Pseudanthias squamipinnis*, 30 *Lysmata amboinensis*, 150 *L. vittata*, 30 *Stenopus hispidus*, and 30 *Urocaridella antonbruunii*, were used to generate the research results of this thesis.

All applicable international, national and institutional guidelines for the care and use of animals were followed. Animal housing was inspected by the James Cook University welfare officer, and ethics approval was granted prior to commencement of this thesis under the James Cook University Ethics Committee Permit numbers A2260 and A2457 (Appendix 1), conforming strictly to the national regulations set out in the National Health and Medical Research Council (2013) Australian code for the care and use of animals for scientific purposes, 8th edition, under Section 39 of the National Health and Medical Research Council Act, 1992.

Wherever possible, fishes (*E. coioides*, *E. lanceolatus* and *L. calcarifer*) that did not require humane euthanasia as an endpoint after experimentation, were quarantined and maintained in holding to be re-used in the Marine Parasitology Laboratory, or were offered for additional teaching practicals for aquaculture and veterinary students, or were offered back to the hatchery from which they were originally sourced. At the end of this thesis, all cleaner shrimp were donated to further study at James Cook University to investigate the closure of their life-cycle and their domestication.

Data storage

All data from which statistical analyses were performed are available through James Cook University electronic data storage through the following permanent links:

Chapter 3: http://dx.doi.org/10.4225/28/5b2c885b32331

Chapter 4: http://dx.doi.org/10.4225/28/5b343a73f35f7

Chapter 5: http://dx.doi.org/10.4225/28/5b344db8591a2

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A note to my wife…

Only you truly know the magnitude of what we have been through together to get to where we are. You have always been my unwavering support and strength. Thank you for supporting me continuously through the rollercoaster of life and throughout my PhD. You have selflessly sacrificed so much of your own aspirations to support mine, and been the cornerstone of our family. I could not have done this without you by my side. I love you and owe you everything.

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As you are currently too young to understand, I hope that in time you might come to consider this message, and the responsibilities that are entrusted to us as parents. You have always been, and will always be the centre of my reasons for taking on the challenges set before me.

Providing you with a safe, stable and prosperous future away from political instability and prejudice has always been my priority. Your mother and I quite literally gave up everything to come to Australia for me to take up this PhD opportunity in the hope of your better future, and we would do anything for you out of the infinite love we have for you.

I was once told by a career guidance counsellor at the end of high school that I would never be able to pursue a scientific degree or career because I had the wrong subject choices. I decided that I wanted to anyway. I had to re-do an additional two years of high school to complete the subjects I had previously missed, and did so part-time while working. My parents could not afford to send me to university so I paid for my tuition and studied to complete my BSc Honours and MSc degrees while working full-time. Education is a privilege. Never let anyone dictate your limits, always foster a good sense of humour in life and have fun, but work hard towards achieving your goals - you will be surprised what you can achieve, and you will learn a lot about yourself in the process.

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with her, but that I would need to put in an application for competitive funding to do so, and fast, as the funding call was about to close. With seven days left before the call closed for applications for the International Post-graduate Research Scholarship, Kate and I (working from Skype and ignoring international time differences) put together the extensive documentation and proposal required for a timely submission, which was ultimately successful.

While my wife and I arranged to liquidate our assets to generate the funds necessary to cover the costs of travel and the associated requirements of our visa, Kate assisted further by taking the time to visit prospective houses we had identified in Townsville to rent, and sent us her opinion on them and photos. Kate also kindly donated her fridge.

As a small token of my gratitude I described a new Australian monogenean (flatworm parasite of fishes) species named in her honour: *Anoplodiscus hutsonae*, which was published in the international journal Systematic Parasitology in 2017. However, I will always be eternally grateful for the opportunity afforded to me by Kate, because it provided so much hope for my family.

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For setting the wheels in motion!

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Abstract

Global aquaculture faces developmental and expansion constraints due to the impacts of diseases. To meet the global demand for protein, aquaculture will need to double by 2050, but a growing concern in aquaculture is the development of drug and chemical resistance by various pathogens, including fish ectoparasites. Of particular concern is the current lack of regulation and surveillance for resistance in the vast majority of aquaculture producing countries, which notably suffer up to 40% production losses due to diseases, at a global combined annual cost exceeding US$6 billion.

Biocontrols such as cleaner fishes have been used successfully in salmonid aquaculture to reduce the impacts of ectoparasitic sea lice, which have traditionally cost this industry millions of US$ annually. This is the only example of exploiting the benefits of a natural cleaning symbiosis for food production. The development of the cleaner fishes biocontrol model was born out of the need to find alternative control measures against the ectoparasitic sea lice which had developed increased levels of resistance to almost all approved commercial drug or chemical applications. The cleaner fishes model is highly successful, and has been developed into its own aquaculture support industry. However, the success of the cleaner fishes model for salmonid aquaculture is restricted geographically, and restricted by the specific feeding preferences of the cleaner fishes to sea lice, and their susceptibility to them.

Tropical and sub-tropical aquaculture experiences a high diversity of economically important fish ectoparasites for which there are no known commercial vaccines, or biocontrols. One of the limiting factors for considering a cleaner fish biocontrol model in tropical and sub-tropical aquaculture is the high probability of susceptibility of cleaner fishes to cosmopolitan ectoparasites with low host-specificity, which infest a large diversity of farmed finfish species.

However, the benefits of cleaning symbiosis in a biocontrol model are likely attainable through the use of cleaner shrimp, which are not susceptible to fish ectoparasites.

Initially, cleaning symbiosis required re-evaluation because it represented two separate but similar mutualisms. Cleaning symbiosis was redefined in Chapter 2 to highlight the importance of predisposing communicative behaviour as the catalyst for true symbiotic cleaning interactions, and a comprehensive global list of cleaners and their distribution was provided. This stabilised the foundation on which to explore questions on the truly symbiotic nature of cleaner shrimp, and their specific cleaning abilities at removing different economically important ectoparasites from cultured fishes and from the environment.

In Chapter 3, the cleaner shrimp *Lysmata amboinensis* was shown unequivocally to tend to injured fish clients in a true cleaning symbiosis, and injury-related inflammation was significantly reduced by the presence of the shrimp. This established the first evidence of wound cleaning of injured fish by cleaner shrimp. The diel ability of *L. amboinensis* was further tested, together with three other shrimp species, *L. vittata*, *Stenopus hispidus*, and *Urocaridella antonbruunii* against three economically important ectoparasites of cultured fish, *Cryptocaryon irritans* (ciliate), *Neobenedenia girellae* (monogenean), and *Zeylanicobdella arugamensis* (leech) for the first time, in Chapter 4. Although all cleaner shrimp reduced ectoparasites, they did so unequally. Of all the cleaners tested, *L. vittata* was considered the superior performer for its ability to reduce parasites by up to 97%. Given the potential of the reduction of reinfective stages in aquaculture by this shrimp's performance, *L. vittata* was selected as the first candidate cleaner shrimp for testing under recirculating aquaculture conditions in Chapter 5 against *N. girellae* infesting cultured grouper, *Epinephelus lanceolatus*.

Lysmata vittata, when used as a biocontrol in Chapter 5, significantly reduced the reinfection of *N. girellae* under recirculating aquaculture conditions by ~87% by consuming the eggs that attach to fish-cage netting. This confirmed the potential of cleaner shrimp to be

used to manage ectoparasites in aquaculture. Ectoparasite benthic stages such as eggs, cysts and cocoons are the traditional source of ectoparasite reinfection in aquaculture, and are impervious to drug and chemical treatments. The use of cleaner shrimp may support the reduction of chemical and drug use to treat parasitic outbreaks on fish, but also offers the first real solution to reducing reinfection pressure by consuming traditionally problematic life-stages, thereby reducing infection severity, translating to improved stock health.

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CHAPTER 1

General introduction

1.1 Global aquaculture and drug resistance

Global aquaculture production volume surpassed captive fisheries in 2013, contributing more food fish for the first time in 2014 (Food and Agriculture Organization of the United Nations (FAO) 2016). Although the statistics and methods used by the FAO to generate these data have recently been questioned (see Pauly and Zeller 2017), the consideration that global aquaculture will generate a significant proportion of the global population's future protein is undeniable. By 2050, aquaculture production will need to double to meet the demand for the world's growing protein requirement, but suggestions of a 'global aquaculture disease crisis' are emerging in the recent literature (Stentiford *et al.* 2017). Diseases are considered the most significant constraint to aquaculture expansion to 2050 (Stentiford *et al.* 2017), but the reasons why are multifaceted. A significant contributor may be the growing trend of pathogen resistance to antimicrobial treatments (Watts *et al.* 2017). In this regard, a parallel can be drawn between this future 'global aquaculture disease crisis' and a concurrent emerging global human health crisis, resulting from general antimicrobial resistance (O'Neill 2014; Watts *et al*. 2017). With the exception of the discussion of, and brief mention in the recent reviews of Done *et al.* (2015) and Watts *et al.* (2017), respectively, these two 'crises', it would seem, currently appear dissociated in the literature, sharing only the same chronological significance. However, up to 80% of all antibiotic drugs find their way into the environment in their active form, resulting in selectively generated drug-resistance (Andersson and Hughes 2014).

Antibiotic discovery dates back to 1928 with the discovery of penicillin by Alexander Fleming, and peaked before 1970 (Davies 2006). However, the development of new drugs globally is in decline, while incidence of antibiotic resistance is increasing (Conly and Johnston

2005). Unsurprisingly, there is "extreme crossover of antibiotic use in human medicine and animal food production", notably in aquaculture (Done *et al.* 2015). This is due partly to the prior existence and availability of these drugs before the global commercialisation of aquaculture around the mid-1980s (Done *et al.* 2015), but also due to the inhibitory costs involved with specific drug development for a developing aquaculture industry (FAO 2017a). Drug resistance in aquaculture is well documented (e.g. McPhearson *et al.* 1991; Tully and McFadden 2000; Fallang *et al.* 2004; Sevatdal *et al.* 2005; Lees *et al.* 2008; Smith 2008; Jones *et al*. 2013; Huang *et al.* 2015; Kathleen *et al.* 2016; Watts *et al*. 2017). However, this documentation is regionally biased, and reflects those countries which have in place the legislation, and the means to enforce the regulation of drugs used in aquaculture, and which incorporate adequate surveillance for drug resistance (Aaen *et al.* 2015; Watts *et al.* 2017). These countries combined contribute approximately only 10% of global aquaculture production, while the remaining 90% is largely unregulated (Done *et al.* 2015; Watts *et al.* 2017), suggesting that our current understanding of drug resistance in aquaculture is grossly exiguous.

But it is not only antibiotics that pathogenic organisms are becoming resistant to. Resistance extends to other drug classes and chemicals used in aquaculture such as avermectins, azamethiphos (organophosphate), pyrethroids, and hydrogen peroxide (Aaen *et al*. 2015), which are typically original agricultural pesticides adapted for use as parasiticides against fish ectoparasites. These drugs and chemicals have been used extensively in salmonid aquaculture against caligid copepod sea lice (Costello *et al*. 2001; Costello 2006, 2009), but other drugs have also been employed against different problematic fish parasites, most notably various anthelmintics such as praziquantel, mebendazole and fenbendazole against endo- and ectoparasitic helminths (e.g. Buchmann and Bjerregaard 1990; Schmahl 1991; Kim *et al.* 1998; Costello *et al.* 2001; Forwood *et al*. 2013; Chagas *et al.* 2016). Praziquantel, the most promising

of these anthelmintics was developed in Germany in 1975 (Day *et al.* 1992) as a treatment against schistosomiasis, the disease caused by members of the flatworm genus *Schistosoma* Weinland, 1858 in humans. It remains the only effective drug against some *Schistosoma* species (King *et al.* 2000), but resistance to the drug was first recorded as little as 19 years later (Fallon and Doenhoff 1994), even though mathematical modelling predicted emergence of resistance in schistosomes to occur only by 2010 (see King *et al.* 2000).

Monogeneans are flatworm parasites of fishes of significant economic importance (see review by Shinn *et al.* 2015), and the main reason why praziquantel has been investigated for use in aquaculture. Praziquantel has been used in aquaculture since the turn of the millennium, and is still largely unregulated and experimental in its application (e.g. Kim *et al*. 1998; Sitjá-Bobadilla *et al*. 2006; Tubbs and Tingle 2006; Williams *et al*. 2007; Yamamoto *et al*. 2011; Iles *et al*. 2012; Forwood *et al*. 2013; Partridge *et al*. 2016). Resistance of monogeneans to praziquantel has not yet been formally reported, although it is suspected for aquaculture farms in South Africa producing *Argyrosomus japonicus* Temminck & Schlegel, 1844 infected with the monogenean *Diplectanum oliveri* Williams, 1989 (K. W. Christison pers. comm.). Whether or not the mechanism of praziquantel resistance in schistosomes and monogeneans would be the same remains unclear, although considered likely to include the involvement of ATP-binding cassette (ABC) transport proteins in schistosomes (Wang and Liang 2012), which are also present in monogeneans (Jones and George 2004).

The emergence of resistance of pathogens to various treatments in aquaculture is likely to have a large impact on aquaculture as it expands into the future, but also on trade in aquaculture commodities. Aquaculture diseases are economically problematic because they negatively influence profitability directly. Financial losses are associated with livestock mortalities, poor livestock growth performance from non-lethal infections, the market rejection of infected livestock (e.g. Ogawa 1994, Moran *et al*. 1999), and the associated costs of disease

mitigation (Lafferty *et al.* 2015). Globally, the financial losses from pathogens are estimated to be approximately 20% of total production value (Sitjá-Bobadilla and Oidtmann 2017), at an estimated cost of ~US$40 billion (FishStatj 2018). Although vaccine development has certainly reduced the need for some antibiotics in aquaculture since the early 1990s, there are no current vaccines available against parasitic agents of fishes (Sommerset *et al*. 2005; Dadar *et al.* 2017).

1.2 The use of cleaner fishes as biocontrols in aquaculture

The problem of drug and chemical resistant ectoparasites in salmonid aquaculture in Europe (Aaen *et al*. 2015), and restrictive drug use regulations (Grave *et al*. 1996) sparked an alternative inventive solution in cleaner fish biocontrols, which has in recent history become a formidable fishery (González and de Boer 2017) and aquaculture-support industry in its own right (see Powell *et al.* 2017). Biocontrols by definition utilise living non-pest organisms to control other pest species (see Treasurer 2002). In European aquaculture, cleaner fishes as biocontrols have proven to be economically viable and cost-effective as a treatment alternative, thus reducing the use of drugs and other chemicals, and the associated risk of resistance (Treasurer 2002). Additionally, cleaner fishes provide a more socially acceptable and environmentally friendly alternative to treatments that can have direct environmental impacts (Powell *et al*. 2017).

Originally, interest in cleaner fish as biocontrols against caligid sea lice infesting Atlantic salmon (*Salmo salar* Linnaeus, 1758) was generated from the pioneering work of Åsmund Bjordal who recorded "cleaning symbiosis between different labrid [wrasse] species and lice infested salmon" in Norway from 1987. The work of Åsmund Bjordal was inspired by the published observations of Potts (1973) (González and de Boer 2017) who observed the wrasses *Symphodus melops* (Linnaeus, 1758) (syn. *Crenilabrus melops*) and *Ctenolabrus rupestris* (Linnaeus, 1758) cleaning various client fish species infested by the parasitic isopod

Gnathia maxillaris (Montagu, 1804) in the former public aquarium at the Marine Biological Association, Plymouth, U.K.

Bjordal's (1988) work was the first to demonstrate what he considered an example of 'cleaning symbiosis' between wrasse and Atlantic salmon, and the first to investigate the use of wrasse as a biocontrol in salmon aquaculture. Bjordal (1988) considered the wrasse species *Centrolabrus exoletus* (Linnaeus, 1758), *Ct. rupestris*, and *Labrus mixtus* Linnaeus, 1758 (syn. *L. ossifagus*) as functional cleaners in salmon aquaculture, and the first full-scale trial was performed in September of the same year (Bjordal 1991). A year later, trials expanded to 20 Norwegian salmon farms and one in Shetland (U.K.), with demonstrated success (Bjordal 1991). Separate trials commenced in Ireland between 1991 and 1992 (Deady *et al.* 1995), and by 1994, 150 000 wrasse had been used in Scotland, and in 1998 over 5 million wrasse were used annually in Norway (Treasurer 2002). Since 2014, the total number of *S. melops* and a fourth species used, *Labrus bergylta* Ascanius, 1767, has exceeded the annual maximum fishing quota of 18 million per species in Norway alone (González and de Boer 2017). Currently, the search for non-drug or chemical based treatments for sea lice control, including the commercial production of cleaner fish species, is considered the most urgent priority for the European Atlantic salmon industry (Powell *et al*. 2017).

The use of cleaner fishes in aquaculture is restricted to the geographic location of available cleaner species. The wrasse species used in Europe are not available in other parts of the world, and early attempts at using other species as cleaners in Canada and Chile failed (Treasurer 2002). Tropical cleaner gobies, native to the Western Central Atlantic region, have also been investigated experimentally for their potential in aquaculture. Cowell *et al.* (1993) tested the efficacy of *Elacatinus genie* (Böhlke & Robins, 1968) and *Elacatinus oceanops* Jordan, 1904 (syn. *Gobiosoma genie* and *G. oceanops*, respectively) against the monogenean *Neobenedenia melleni* (MacCallum, 1927) on seawater-cultured hybrid tilapia in the Bahamas.

The same parasite was the focus of another study using *Elacatinus figaro* Sazima, Moura & Rosa, 1997 as a cleaner for *Epinephelus marginatus* (Lowe, 1834) in Brazil (de Souza *et al*. 2014). *Elacatinus oceanops* has also been used experimentally with cultured *Lutjanus analis* (Cuvier, 1828), *Rachycentron canadum* (Linnaeus, 1766), and *Seriola dumerili* (Risso, 1810) (Benetti *et al*. 2007).

1.3 Cleaning symbiosis, and its ambivalence

The use of cleaner fishes as biocontrols employs what is considered as 'cleaning symbiosis' (Bjordal 1988, 1992), an interspecific mutualism. Cleaning symbiosis has been well documented between fishes since the early 1960s, thanks in part to the popular text of Limbaugh (1961). In his publication "Cleaning symbiosis" in Scientific American, Conrad Limbaugh described in great detail and with great enthusiasm, his observations made while diving in the late 1940s and mid-1950s. His work was met posthumously with a mixed response from the scientific community, from unquestioned acceptance (e.g. Gotshall 1967) to criticism (Hobson 1969, 1971; Losey 1972; Gorlick *et al.* 1978), and even blatant sarcasm (Spotte 1998). Regardless, the observations of Limbaugh and his bold interpretations thereof, spawned constructive debate, and a renewed scientific interest in the subject globally, which continues to this day.

Cleaning symbiosis was officially defined by Howard Feder in 1966 as "*the removal of ectoparasites, bacteria, diseased and injured tissue, and unwanted food particles by cleaner organisms from cooperative host* [= client] *organisms*". Since then, publications on cleaning symbiosis have increased steadily (Fig. 1), at an average rate of about 4.3 articles per year over the last 50 years. In an additional remark, Feder (1966) added that the mutually beneficial behaviour between the client and cleaner provides a food source for the cleaner. This remark has been assumed by many to mean that the consumption of parasites and other material by a

'cleaner' organism from another organism (i.e. the act of cleaning) is synonymous with 'cleaning symbiosis' (e.g. Losey 1972; Hobson 1976; Swartz 1981; Gorlick *et al*. 1987; Bjordal 1988; Poulin and Vickery 1995; Carvalho *et al.* 2003; Krajewski 2007; Cheney *et al*. 2009; Lee *et al*. 2009; Sazima 2010; Brown *et al*. 2012, Huebner and Chadwick 2012a; Farrell *et al*. 2014; Militz and Hutson 2015; Morais *et al.* 2016). However, the current definition blurs the boundaries between two separate mutualisms.

Isabelle Côté was the first to recognise two distinct categories of cleaning symbiosis (Côté 2000). The first included 'incidental cleaning', which reflected no particular evolutionary adaptations to a cleaning role by a cleaner, and contained examples between animals where algae and other epibionts are simply removed from one animal by another as they would be from any other substrate, in an opportunistic manner (Côté 2000). Such examples include the interactions between *Atherinops affinis* (Ayres, 1860) and gray whale, *Eschrichtius robustus* (Lilljeborg, 1861) (see Swartz 1981), *Chlorophthalmus agassizi* Bonaparte, 1840 and tunicate *Aplidium* Savigny, 1816 sp. (syn. *Amaroucium* Milne Edwards, 1841) (see Rathjen 1960), echinoderms and sponges, copepods and tunicates (Hendler 1984; Perissinotto and Pakhomov 1997, in Côté 2000), *Planes minutus* (Linnaeus, 1758) and loggerhead turtle, *Caretta caretta* (Linnaeus, 1758) (Davenport 1994), freshwater crayfish and branchiobdellids (Brown *et al.* 2002; 2012; Ames *et al*. 2015), the anemone, *Anthopleura hermaphroditica* (Carlgren, 1899) (syn. *A. aureoradiata*) and cockle, *Austrovenus stutchburyi* (W. Wood, 1828) (see Mouritsen and Poulin 2003), and juvenile *Bodianus anthiodes* (Bennett, 1832) and crinoids (Schiaparelli and Alvaro 2009). The second, more common category reflected an evolutionary process driving specific behavioural and morphological adaptations to support a cleaning lifestyle (Côté 2000). Examples include the most familiar in the literature, such as the cleaner wrasse *Labroides dimidiatus* (Valenciennes, 1839).

Howard Feder's "*cooperative host organisms*" appears to have been neglected to some extent, or the lack of qualification for "*cooperative*" has resulted in its consideration by researchers in all its perceivable extremes. However, the removal of algae and other epibionts "from any suitable substrate" (Côté 2000) does not qualify as cooperation. To cooperate, organisms need to be cognitive of intent, and must be able to act between the choice to accept or to reject (Axelrod 1984). Therefore currently, cleaning symbiosis is ambivalent and contains examples of another separate opportunistic incidental mutualism, referred to as incidental cleaning (Côté 2000). Indeed, cleaning symbiosis has also been completely misrepresented in the literature in terms of decomposing animal wastes and ectomycorrhizal fungi (see Sagara 1995).

True cleaning symbiosis therefore implies cooperation from both parties, not just the client, and is preceded by visual signalling or by tactile stimulation (communication), which reflects the intent, and generates the reciprocal response though choice (see examples in Limbaugh 1961; Tyler 1963; McCutcheon and McCutcheon 1964; Feder 1966; Youngbluth 1968; Abel 1971; Able 1976; Ayling and Grace 1971; Hobson 1971, 1976; Losey 1972, 1974, 1979; Wyman and Ward 1972; MacFarland and Reeder 1974; Sargent and Wagenbach 1975; Sulak 1975; Brockmann and Hailman 1976; Corredor 1978; Minshull 1985; Sikkel 1986; Stauffer 1991; Soto *et al.* 1994; Van Tassell *et al*. 1994; Galeote and Otero 1998; Wicksten 1995, 1998; Poulin and Grutter 1996; Sazima *et al.* 1998a; Sazima *et al.* 2005; Côté 2000; Shigeta *et al*. 2001; Sazima and Moura 2000; Sazima and Sazima 2000; Becker *et al.* 2005; Shepherd *et al.* 2005; Craig 2007; Bertoncini *et al*. 2009; Horton 2011; Abe *et al.* 2012; Huebner and Chadwick 2012a; Karplus 2014; Chapter 2).

Terms of intimacy are used in the cleaning symbiosis literature to reflect the degree of the relationship between cleaners and clients, categorising them into two distinct groups. Both 'facultative' and 'obligate' cleaners have traditionally been recognised; the former includes

cleaners which demonstrate less reliance on cleaning symbiosis as a way of life, but that interact with clients as opportunities present themselves, and quite notably, but not exclusively, as juveniles (Côté 2000). The term 'obligate' is inaccurate, as is the implication that these cleaners rely solely on cleaning symbiosis for their source of food. These cleaners demonstrate a more dedicated cleaning lifestyle than facultative cleaners, and many familiar examples include those species which tend prominent cleaning stations on the reef which are frequented by clients (Limbaugh 1961). Both categories of cleaners contain aquatic members with prominent markings, which persist throughout their non-larval ontogeny, or are observed only in juveniles.

1.4 Cleaner guild hypothesis

The similarity in colours and markings in different cleaners has generated debate since the cleaner guild mark hypothesis was proposed by Eibl-Eibesfeldt (1955). One of the earliest suggestions was that a prominent black lateral stripe present in both *L. dimidiatus* and *E. oceanops*, representing two separate fish families in separate geographic regions, had evolved to draw attention to their cleaner status (Eibl-Eibesfeldt 1955; Potts 1973). This state represented tropical cleaners, but was apparently less dramatic in temperate species, advertised rather by a black spot on the caudal peduncle or caudal fin (Potts 1968, 1973). The guild mark phenomenon was further supported by observations made by Ayling and Grace (1971) of New Zealand facultative cleaner wrasses which displayed either prominent lateral stripes, or a black caudal bar (Ayling and Grace 1971). These guild marks were considered by Ayling and Grace (1971) to function as visual conspicuous contrast and therefore as an attracting signal to client fishes, but the presence of both guild "types" in temperate cleaner fishes off New Zealand reflected the "modified subtropical fauna" (Ayling and Grace 1971). Later, Côté (2000) could only find partial support for the guild mark hypothesis, which was based on her analyses

supporting the hypothesis in "obligate" cleaners, but suggested no significant difference between patterns on facultative cleaners and non-cleaners. However, these analyses were flawed, as they compared intra-generically for "obligate" cleaners (*Elacatinus* Ginsburg, 1944 spp.), yet inter-generically for facultative cleaners (see Appendix 1 of Côté 2000). In addition, at least one non-cleaner listed (*B. anthiodes*) would ultimately be shown to be a juvenile facultative cleaner (Bshary 2003). The debate and interest in the guild mark hypothesis was biased at this point towards marine species until it was considered for the first time for the freshwater catfish *Platydoras costatus* (Linnaeus, 1758) by Carvalho *et al*. (2003). Juveniles of this species are facultative cleaners, and also have strongly contrasting lateral black and white striping (Carvalho *et al.* 2003).

Lateral black striping was later demonstrated experimentally by Stummer *et al.* (2004) to act as a long-distance signal of cleaner fish status, attracting clients; the longer the stripe, the more initial attention it attracted (Stummer *et al*. 2004). This to some degree explained why striped "obligate" cleaner fishes (*Elacatinus* spp.) have a longer stripe than their non-cleaning congeners (Côté 2000), but lateral striping in fishes has also evolved in associated with a predatory (piscivorous) lifestyle, and as a prey defence mechanism, interfering visually in the perception of prey by predators (Seehausen *et al.* 1999). How then do client species tell the difference? A partial explanation may be offered by Côté (2000) and Stummer *et al.* (2004), who demonstrated that cleaners are smaller in size than non-cleaners (Côté 2000), and that lateral striping is more significant for client-cleaner communication on a smaller cleaner body size (Stummer *et al*. 2004). Predators are generally larger than their prey items, and lateral striping as a defence mechanism is associated with largely shoaling species (Seehausen *et al.* 1999), thereby acting to disrupt the profile of individuals. Cleaners generally are smaller than their clients, and are not known to congregate in dense shoals. Most operate in small groups (e.g. Limbaugh 1961; Feder 1966), in pairs, or singly (e.g. Ayling and Grace 1971).

In their phylogenetic analysis of wrasse, Arnal *et al*. (2006) found no evidence for the relationship between body size and cleaning behaviour. Both Côté (2000) and Arnal *et al.* (2006) used maximum body length of adults for each of their species in their tabulated lists (see also Table 1 of Arnal *et al*. 2006, *cf.* FishBase, Froese and Pauly 2017), but the majority of species listed are facultative cleaner wrasses which clean only as juveniles (Côté 2000). All wrasses are protogynous hermaphrodites, the majority of which change colour and colour pattern as they grow and change phase (Heemstra and Heemstra 2004). Therefore the guild markings present in juveniles, or even between sexes may not be the same in adults of the same species, or may not be present in adults at all. This potential confounding was not considered in the analysis of Arnal *et al*. (2006). In addition, recent evidence provided by Baliga and Mehta (2014) also indicated that other morphological traits related to feeding function coincide with ontogenetic shifts away from cleaning behaviour in these facultative cleaners. Therefore the consideration of Stummer *et al.* (2004) that the significance of lateral striping is body size dependant, is probably the most accurate.

Arnal *et al.* (2006) were the first to demonstrate an evolutionary link between cleaning and fish colour, which was initially thought to involve short-distance signalling (Stummer *et al.* 2004). Cheney *et al.* (2009) demonstrated that both colour and pattern were important in cleaner fish signals. In their analyses, cleaner fishes were more likely to display both blue and yellow body colouration compared to non-cleaners, but that blue was one of the most conspicuous colours against the coral reef background. This was tested against three different fish visual systems including sensitivity to ultraviolet (<400 nm; Cheney *et al*. 2009), suggesting that blue colouration may serve as a longer-distance signal. In their analyses, yellow was the most effective contrasting colour against black body patterns, and the blue of the water background, but a combination of yellow, black and blue on a cleaner conveyed maximum visibility (Cheney *et al.* 2009).

To date, all work done on the cleaner guild hypothesis has been on cleaner fishes, most of which inhabit shallow inshore coral reef systems. This hypothesis has not been formally investigated for cleaner shrimp*, although some cleaner shrimp species also display prominent markings, including contrasting lateral stripes or spots. Many contrasting patterns in cleaner shrimp include red against white, not black as in cleaner fishes. The colour white in cleaner shrimp may serve as an indication of their cleaning status. Many cleaner shrimp species possess white antennal or antennual flagella (Karplus 2014), white appendages, or a white mid-dorsal stripe (Wicksten 2009). The white colour of these structures was considered a necessary feature for a shrimp to be considered a cleaner (Wicksten 2009), which is also partly supported by Bruce (1976) who mentioned that non-cleaners rarely possess this type of colouration. However, not all cleaner shrimp have white antennal or antennual flagella (e.g. *Stenopus tenuirostris* de Mann, 1888, and *Urocaridella antonbruunii* (Bruce, 1967) (Palaemonidae; Calado 2008), which might indicate that the colour white serves more for visual recognition during the day. Karplus (2014) emphasised the potential lucidity of red and white alternating colouration in various cleaner shrimp and that a study of the sensitivity of fishes to this colour pattern should be investigated. However, longer wavelengths such as red and yellow attenuate first with depth in seawater, thus red appears dark or black with increased depth. Bright colouration may serve a signalling function in cleaner shrimp found in shallow waters, but if a "cleaner blue" guild as proposed by Cheney *et al*. (2009) holds true for cleaner shrimp, it may be expected that deeper water cleaner shrimp would also reflect one of the blue categories discussed by them, or that ultraviolet might be used to signal a specific clientele sensitive to this spectrum. This remains to be tested*.

*The cleaner guild hypothesis and reflectance wavelength spectrum of shrimp was tested, and is presented in Appendix 6, but falls outside of the main thesis investigation due to limited practicality of data collection in wild observations.

1.5 Cleaner diversity

At the time of his publication, Limbaugh (1961) mentioned that 26 fishes, 6 shrimp and a crab were considered as cleaners. This number grew to 112 fishes and 20 crustaceans by 1994 (Van Tassell *et al*. 1994). Thereafter, Côté (2000) published her review listing 107 fishes and 24 crustaceans. The review of Côté (2000) excluded freshwater taxa, and any reports of cleaners from captivity, but augmented the list constructed of marine species by Van Tassell *et al.* (1994). However, 14 records of marine cleaner fishes, and 4 cleaner shrimp were missed at that time by both reviews, as well as 2 freshwater cleaner fishes by Van Tassell *et al.* (1994). In addition, some marine fishes listed by Van Tassell *et al.* (1994) were also excluded without comment, or in error by Côté (2000), e.g. *Siphamia tubifer* Weber, 1909, and *Atypichthys strigatus* (Günther, 1860). Since both reviews, 8 additional freshwater fishes, 54 additional marine fishes, and 26 additional cleaner shrimp have been identified as cleaners in the primary literature. Currently the use of the term 'cleaner shrimp' is used ambiguously in the literature, suggesting incorrectly that cleaner shrimp exceed 175 species (e.g. Martinelli-Filho *et al.* 2008). In addition to this, many of the cleaner species listed in previous literature are taxonomically out-dated. A list of globally known cleaner species is a valuable resource that could provide information on what potential cleaners could be explored and used in aquaculture applications in different parts of the world. To accomplish this, cleaning symbiosis and the associated literature require formal revision to address inconsistencies, and to update the current knowledge of both cleaner fishes and shrimp species known to clean (Chapter 2).

1.6 Cleaner shrimp and their potential as biocontrols in aquaculture

Cleaner shrimp have historically been given less attention than cleaner fishes. Although discussed by Limbaugh (1961), Limbaugh *et al*. (1961), and others, comparatively few records of cleaner shrimp as cleaners persist in the historic cleaning symbiosis literature. One of the

reasons is that many cleaner shrimp species are cryptic (Huebner and Chadwick 2012a), often nocturnal, and crevice-dwelling (Huebner and Chadwick 2012b), which makes them difficult to observe compared to the comparatively charismatic nature of many cleaner fish species. As a result, their ability as cleaners was based on speculative accounts for nearly 40 years (e.g. Chace 1958; Randall 1958; Limbaugh 1961; Limbaugh *et al.* 1961; Feder 1966; Sargent and Wagenbach 1975; Criales and Corridor 1977; McCourt and Thomson 1984; Jonasson 1987; Wicksten 1995), and their cleaner status was vehemently opposed by student and seasoned mainstream marine researchers alike (Turnbull 1981; Spotte 1998, respectively). But just as opponents of the 40-year cleaner-shrimp dogma were making their case for conjecture, the first empirical evidence for cleaning by a cleaner shrimp was published by Bunkley-Williams and Williams (1998), and ironically, the ethology of the same species, *Ancylomenes pedersoni* (Chace, 1958) was used to justify opposing arguments.

Ancylomenes pedersoni was observed by Limbaugh (1961) removing parasites from client fishes, and was considered capable of removing subcutaneous parasites by direct manipulation of overlying and surrounding skin (Limbaugh 1961; Limbaugh *et al.* 1961). However, no verifiable evidence was available in support of the claims made by these authors. Additionally, Turnbull (1981) could not provide any evidence from exhaustive observations that *A. pedersoni* removed conspicuous crustacean ectoparasites of fishes in the wild, and no parasite remnants were recovered by Turnbull (1981) in shrimp stomach analyses. This was considered by Spotte (1998) as grounds for dismissing cleaner shrimp as cleaners. However, the latter observations were contradicted when *A. pedersoni* immediately removed and ate all juvenile isopod parasites, *Anilocra haemuli* Williams & Williams, 1981 when infected *Haemulon flavolineatum* (Desmarest, 1823) were introduced into an aquarium housing them (Bunkley-Williams and Williams 1998). Three other cleaner shrimp species were used in the study by Bunkley-Williams and Williams (1998), namely *Lysmata grabhami* (Gordon, 1935),

Stenopus hispidus (Olivier, 1811), and *Stenopus scutellatus* (Rankin, 1898), but none of these species removed any parasites.

As a renewed interest in the abilities of cleaner shrimp was sparked by the results of Bunkley-Williams and Williams (1998), four additional empirical studies were published over the following 17 years demonstrating the ability of cleaner shrimp species to remove fish ectoparasites (Becker and Grutter 2004; Östlund-Nilsson *et al.* 2005; McCammon *et al*. 2010), or to consume parasite environmental stages (Militz and Hutson 2015). Combined, these five studies considered only 10 shrimp species, of which only four demonstrated a significant reduction of live parasite numbers (Table 1).

The contradictions demonstrated by the studies of the same shrimp species by Turnbull (1981) and Bunkley-Williams and Williams (1998), and the lack of performance against the same parasites by other cleaner shrimp species, is suggestive of an alternative hypothesis that not all cleaner shrimp perform parasite removal equally; that some shrimp may be specialised to remove or consume non-crustacean or non-helminth parasites, or only specific life-stages thereof (*cf.* Table 1), and that parasites are not the only proximate cause of cleaning by cleaner shrimp. Indeed, the consideration that shrimp which do not remove or consume parasites are therefore not cleaner shrimp (Turnbull 1981; Bunkley-Williams and Williams 1998; Spotte 1998; McCammon *et al.* 2010), is a false dilemma. *Stenopus hispidus* is a poor performer of fish parasite reduction (Table 1), and has been questioned as a cleaner shrimp due to this specific lack of performance (see Bunkley-Williams and Williams 1998; McCammon *et al*. 2010). However, this shrimp species has been observed cleaning sea turtles of epibionts and possibly dead skin (Sazima *et al.* 2004a). Indeed other cleaning functions may exist which have never been explored for cleaner shrimp, but have been alluded to in the literature.

Titus *et al*. (2015) identified a ‘cleaning discordance’ between cleaner shrimp and fish, evident by the lack of competition between shrimp and fish cleaners for the same clients. Titus

et al. (2015) suggested that one explanation might be that these cleaners might offer slightly different cleaning services. Some shrimp may function as cleaners of wounds or injuries (Limbaugh 1961; Corredor 1978; Crump 2009), similar to cleaning of injured fishes on a Barbadian reef by cleaner fishes (Foster 1985). Shrimp may also tend bacterial infections (Limbaugh 1961). The possible function of cleaner shrimp as wound cleaners, or their ability to influence wound healing, is yet to be investigated and tested empirically.

Recently, Militz and Hutson (2015) demonstrated for the first time the important ecological function of the cleaner shrimp *Lysmata amboinensis* (de Man, 1888) as a non-symbiotic cleaner. By foraging and consuming the environmental life-stages (eggs and oncomiracidia) of the parasite *N. girellae*, *L. amboinensis* reduced reinfection pressure (Militz and Hutson 2015). Therefore, cleaner shrimp could influence parasite numbers without direct contact with client fish. These authors suggested that as a result, cleaner shrimp may offer an alternative solution as a biocontrol against certain parasites in tropical aquaculture (Militz and Hutson 2015).

No biocontrols against fish parasites are currently employed in tropical aquaculture, although parasitic diseases may account for between 30% and 50% of aquaculture stock losses in parts of the Asia-Pacific region (Shinn *et al.* 2015). One of the main reasons biocontrols against fish parasites are not employed in this region is the availability of largely unregulated drugs and chemicals, and a lack of clear or effective drug-use restrictions driving little need to explore alternative control measures for parasitic diseases. However, as countries in this region develop towards a higher level of responsible practice in aquaculture to meet international initiatives such as the The Global Plan of Action on Antimicrobial Resistance (FAO 2017a), attention will need to shift to future alternative control strategies, as was the case previously in Europe.

Cleaner shrimp as biocontrols could certainly provide potential advantages beyond the limitations of cleaner fishes. Although cleaner shrimp may be able to transmit bacteria and viruses, they are not susceptible to fish ectoparasites. Therefore, cleaner shrimp cannot transmit them to the cultured client fish stock they would be employed to clean, unlike cleaner fishes which may pose a risk of potential transmission of parasites to these clients. Conversely, cleaner fishes may also face the risk of being infected by parasites directly from infected clients. The European cleaner wrasse success story is largely due to their insusceptibility to the various caligid sea lice which they are employed to remove from cultured salmonids. However, recent research has highlighted concerns that lumpfish (*Cyclopterus lumpus* Linnaeus, 1758) is parasitised by at least one of these sea lice species (*Caligus elongatus* von Nordmann, 1832), and both the wrasse species and lumpfish are susceptible to, and can transmit *Paramoeba perurans* (Young, Crosbie, Adams, Nowak, and Morison, 2007) *sensu* Feehan *et al.* (2013), the aetiological agent of amoebic gill disease, an emerging disease of salmonids in Europe (Karlsbakk *et al*. 2013, 2014, Karlsbakk 2015; Haugland *et al.* 2017; Powell *et al*. 2017). *Paramoeba perurans* is pathogenic in many aquacultured fish species globally (Haugland *et al.* 2017), but remains primarily a problem for cold water species.

Parasites of fishes that are already problematic in tropical aquaculture, and that have a low host-specificity and a broad geographic distribution such as *Amyloodinium ocellatum* (E. Brown) E. Brown & Hovasse, 1946, *Cryptocaryon irritans* Brown, 1951, *Neobenedenia* Yamaguti, 1963 spp., and *Zeylanicobdella arugamensis* de Silva, 1963 etc. (de Silva 1963; Ogawa *et al.* 1995; Cruz-Lacierda *et al.* 2000; Shinn *et al*. 2015; Mo *et al.* 2016) are more likely to pose a risk for cleaner fishes in tropical aquaculture. In addition, the diversity of cultured fish species is far greater in the Asia-Pacific region that it is in Europe (*cf.* FAO 2014, 2017b). Therefore there could be greater potential risk of parasitic disease emergence in cleaner fishes if they were employed in the tropics.

Approximately 20 known tropical cleaner shrimp species are found in the Asia-Pacific region (see examples in Holthuis 1946; Corredor 1978; Baensch and Debelius 1992; Debelius 1999; Côté 2000; Becker and Grutter 2004; Becker *et al.* 2005; Okuno 2005; Calado 2008; Baeza 2009; Okuno and Bruce 2010; Hou *et al.* 2013; Karplus 2014). To determine whether any of these species would be a viable biocontrol agent against fish parasites, initial exploratory research similar to that done by Åsmund Bjordal in the 1980s on European cleaner wrasse, would need to be investigated. Prospective cleaner shrimp would need to be tested for their efficacy against economically important target parasites, both symbiotically (i.e. with client fishes), and non-symbiotically (i.e. against environmental life-stages; Militz and Hutson 2015). Any potential candidate cleaner shrimp species identified thereafter would need further testing to demonstrate efficacy under aquaculture or simulated aquaculture conditions (see Bjordal 1988). An ideal candidate would also need to be resilient, easily accessible, widely distributed, and preferably easy to culture in captivity.

1.7 Research aims / hypotheses

This thesis presents four distinct research aims which are presented as a series of consecutive thesis chapters:

1. A review of the cleaning symbiosis literature, and an amendment of the original definition to separate cleaning symbiosis from the separate mutualism which is incidental cleaning (Chapter 2). Based on the amended definition, a comprehensive list of all globally known cleaner fishes and crustaceans is generated which is inclusive of freshwater and marine taxa, and is taxonomically current.
2. The first investigation of cleaner shrimp as wound cleaners of injured fish, testing the hypotheses that cleaner shrimp do attend to these injuries; that these interactions reflect

a truly cleaning symbiotic interaction, and that cleaner shrimp influence the wound healing process (Chapter 3).

3. The different specific and temporal abilities of four cleaner shrimp species to remove and to consume the parasitic stage (on client fish) and the environmental stages of three economically important ectoparasites of tropical aquaculture (Chapter 4). Here, the hypothesis that not all cleaner shrimp clean equally, and that different species demonstrate different cleaning preferences, is tested. In addition, from the results obtained, I aimed to identify a potential candidate cleaner shrimp species for further testing under simulated aquaculture conditions.
4. The efficacy of the candidate cleaner shrimp species (from 3 above) against the parasite *Neobenedenia girellae* infecting cultured grouper under simulated aquaculture conditions, testing the hypothesis that cleaner shrimp are effective biocontrols (Chapter 5).

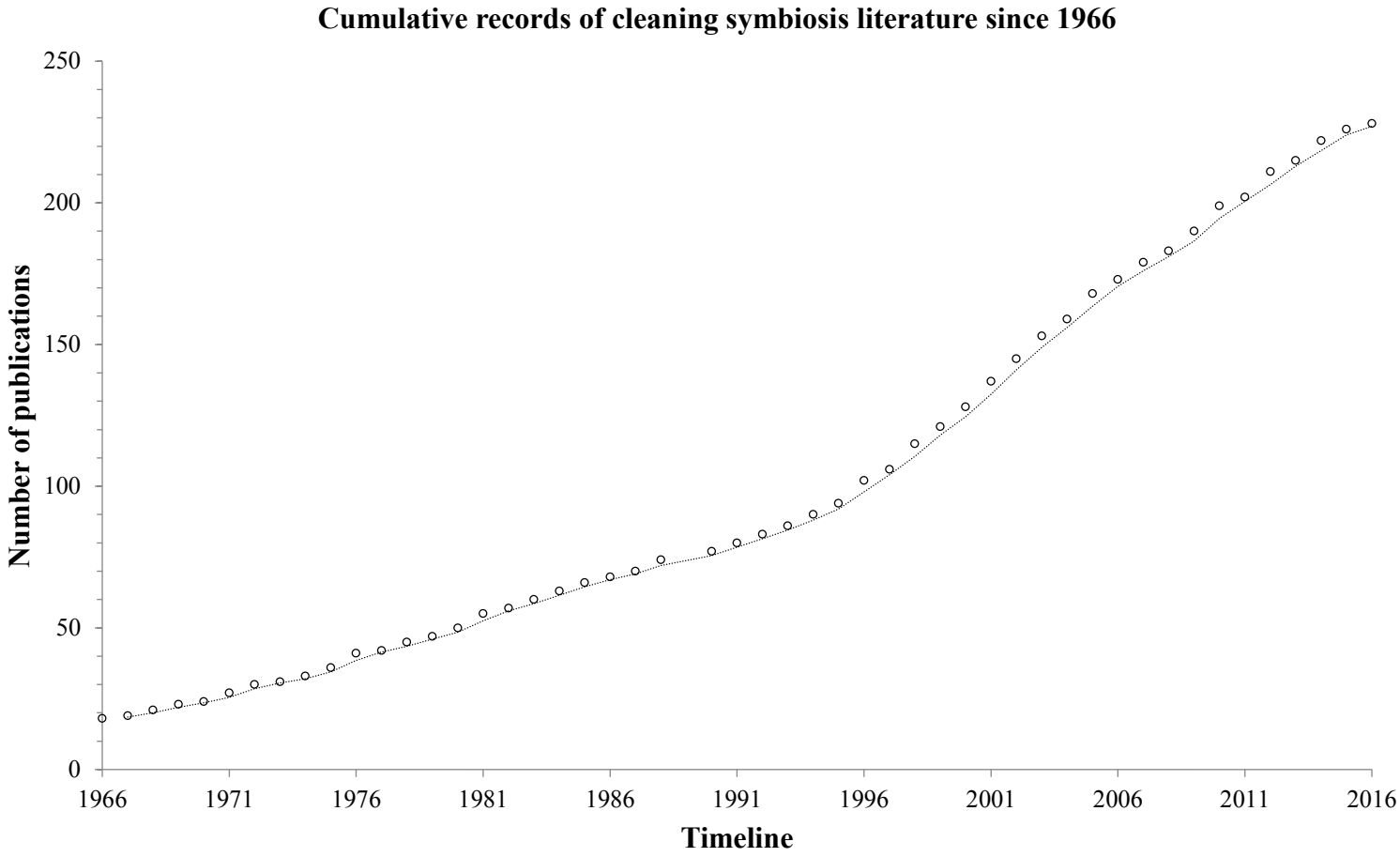

Fig. 1.1. Approximate cumulative records of aquatic cleaning symbiosis since the original definition of Feder (1966).

Table. 1.1. Empirical studies demonstrating significant parasite reduction by cleaner shrimp species

Cleaner shrimp	Parasite	Significantly reduced	References
Ancylomenes holthuisi (Bruce, 1969)	*Benendenia* Diesing, 1858 sp.	Yes	Becker and Grutter (2004)
	Juvenile Gnathiidae and larval Copepoda	Wild gut analyses only	Becker and Grutter (2004)
Ancylomenes pedersoni (Chace, 1958)	Juvenile *Anilocra haemuli* Williams & Williams, 1981	Yes	Bunkley-Williams and Williams (1998)
	Neobenedenia pargueraensis Dyer, Williams & Bunkley-Williams, 1992[1]	Yes	McCammon *et al.* (2010); Loerch *et al.* (2015)
Lysmata amboinensis (de Man, 1888)	*Neobenedenia* Yamaguti, 1963 sp. (*Neobenedenia girellae* (Hargis, 1955))[2]	Yes[3]	Militz and Hutson (2015)
Lysmata grabhami (Gordon, 1935)	Juvenile *Anilocra haemuli*	No	Bunkley-Williams and Williams (1998)
Palaemon adspersus Rathke, 1837	*Gyrodactylus* von Nordmann, 1832 spp.	Yes	Östlund-Nilsson *et al.* (2005)
	Lepeophtheirus pectoralis (Müller O.F., 1776)	Not experimentally verified[4]	Östlund-Nilsson *et al.* (2005)
Palaemon elegans Rathke, 1837	*Gyrodactylus* spp.	Not experimentally verified[5]	Östlund-Nilsson *et al.* (2005)
Periclimenes yucatanicus (Ives, 1891)	*Neobenedenia pargueraensis*	No	McCammon *et al.* (2010); Loerch *et al.* (2015)
Stenopus hispidus (Olivier, 1811)	Juvenile *Anilocra haemuli*	No	Bunkley-Williams and Williams (1998)
	Neobenedenia pargueraensis	No	McCammon *et al.* (2010); Loerch *et al.* (2015)
Stenopus scutellatus (Rankin, 1898)	*Neobenedenia melleni* (MacCallum, 1927)	No	Bunkley-Williams and Williams (1998)
Urocaridella Borradaile, 1915 sp. c	*Benendenia* Diesing, 1858 sp.	Not experimentally verified[6]	Becker and Grutter (2004)
	Juvenile Gnathiidae and larval Copepoda	Wild gut analyses only	Becker and Grutter (2004)

1. The species named in McCammon *et al.* (2010) is *Neobenedenia melleni*, but likely in error after reference to *N. pargueraensis* in Loerch *et al*. (2015) for the same parasite and research; 2. *Neobenedenia* sp. of Militz and Hutson (2015) = *N. girellae* of Brazenor *et al.* (2018); 3. Demonstrated reduction of environmental stages and reinfection success; 4. Single observation only; 5. Not tested for its efficacy to remove *Gyrodactylus* spp., only observed feeding on the host fish; 6. Not tested for its ability to remove *Benedenia* sp. off host fish.

CHAPTER 2

Cleaner fishes and shrimp diversity and a re-evaluation of cleaning symbioses

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In this chapter, I address the ambivalence of the original cleaning symbiosis definition, re-evalaute cleaning symbiosis, and explore the diversity of aquatic cleaning organisms from both marine and freshwater environments, to address the first thesis aim.

2.1 Abstract

Cleaning symbiosis has been documented extensively in the marine environment over the past 50 years. The global cleaner diversity comprises an estimated 208 fish species from 106 genera representing 36 families and 51 shrimp species from 11 genera representing six families. Cleaning symbiosis as originally defined, is amended to highlight communication between client and cleaner as the catalyst for cooperation, and to separate cleaning symbiosis from incidental cleaning, which is a separate mutualism preceded by no communication. Moreover, the term ‘dedicated’ is proposed to replace ‘obligate’ to describe a committed cleaning lifestyle.

Marine cleaner fishes have dominated the cleaning symbiosis literature, with comparatively little focus given to shrimp. The engagement of shrimp in cleaning activities has been considered contentious because there is little empirical evidence. Plasticity exists in the use of 'cleaner shrimp' in the current literature, with the potential to cause significant confusion. Indeed, this term has been used incorrectly for the shrimp Infraorder Stenopodidea, involving three families, Stenopodidae, Palaemonidae, and Hippolytidae, and to represent all members of *Lysmata* Risso, 1816 and *Stenopus* Olivier, 1811. Caution is expressed in the use of grey literature and anecdotal observations to generate data on cleaning interactions, due to the presence of species complexes. Interest in cleaning organisms as biological controls in aquaculture is increasing due to their value as an alternative to various chemical ectoparasite controls. Reports of the importance of cleaner organisms in maintaining a healthy reef ecosystem have also been increasing and the current biological knowledge on cleaner organisms is reviewed, highlighting areas that are understudied.

2.2 Introduction

Symbiosis is the living together of two or more different taxa, and includes mutualism, parasitism and commensalism (Martin and Schwab 2013; Fig 2.1). However, many symbiotic relationships are subtle, and the variables that influence them can often be overlooked (Feder 1966; Egerton 2015), or have been incorrectly interpreted. The term symbiosis is considered by some authors to include only those interactions in which both symbionts live together in prolonged intimate contact, or where these symbionts are physiologically integrated (Bauer 2004; Bronstein 2015). As such, the temporary mutualism representing cleaning symbiosis is considered by these authors as non-symbiotic. However, de Bary (1879) discussed less permanent symbiotic interactions (Peacock 2011; review by Martin and Schwab 2013). Peacock (2011) labelled the notion of 'intimate contact' as imprecise and too restrictive

because it is highly scale-dependent. He added that there are casual interactions between symbionts. The term 'cleaning symbiosis' has thus become widely used in the literature with over 1,000 hits in Google Scholar. In this chapter I follow the view of Peacock (2011) that cleaning symbiosis reflects a legitimate symbiosis.

Cleaning symbiosis was defined by Feder (1966) as the removal of ectoparasites, bacteria, diseased and injured tissue, and unwanted food particles by cleaner organisms from cooperative host organisms. Feder (1966) added that the mutually beneficial behaviour also provides a source of food for the cleaner. Losey (1972) added 'and subsequent ingestion' to emphasise this nutritional benefit for the cleaner. However, the original definition is in need of amendment because it excludes communication as the catalyst for cooperation in these interactions and does not clearly highlight the shared reason for this cooperation; it presents a positive effect on the survival of both client and cleaner.

The use of imprecise terminology in the biological sciences is common (Wilkins 2005). The frequent misuse or misinterpretation of terms such as "cleaning symbiosis" or "cleaner shrimp" over the last 20 years has created significant ambiguity in the literature. The construction of terms of intimacy to attempt to further qualify the degree of the cleaning relationship has created further ambiguity. For example, the term 'obligate' denotes a strict necessity in its mode, outside of which survival is compromised. In the cleaning symbiosis literature, the term 'obligate' is used for a lack of a term to describe a semi-permanent or full-time cleaner organism. Yet, cleaners can live independently, thus no cleaners are obligate.

This review provides the first taxonomically updated global estimate of cleaner fishes and shrimp diversity. Furthermore the inconsistencies and ambiguity in the relevant literature are addressed, to refine the definition of a cleaning symbiosis, and to explore the attributes that define cleaner organisms. This is the first review to separate incidental cleaning from cleaning

symbiosis. The review of Côté (2000) is expanded on to include freshwater species and those fishes and shrimp newly identified as cleaners.

2.3 Cleaning symbiosis

The first possible recorded observation of a cleaning symbiosis between two different species was made by the Greek historian Herodotos in the fifth century BC. Herodotos observed the cleaning interaction between a bird he called 'the trochilus' (not to be confused with the hummingbird genus *Throchilus* Linnaeus, 1758) and a Nile crocodile (*Crocodylus niloticus* Laurenti, 1768) which allowed the bird access to its mouth to remove leeches (Herodotos). Although cleaning symbioses are reported from terrestrial ecosystems (e.g. Hart *et al.* 1990; Mooring and Mundy 1996; Sazima *et al.* 2012), they appear to be more common and diverse in aquatic environments, particularly in tropical marine environments (Limbaugh 1961; Poulin and Grutter 1996; Grutter 2002). The greater number of observations in tropical aquatic versus temperate aquatic environments may reflect greater visibility underwater, higher species richness, as well as biogeographic and habitat distributions of client and cleaner species. The majority of published reports on cleaning symbioses from aquatic environments deal with fishes as cleaners (Table 2.1). Marine crustaceans as cleaning organisms (Table 2.2) have received far less attention historically, partly due to their often cryptic crevice-living nature. There are currently no reports of cleaning interactions involving freshwater crustaceans. However, cleaner shrimp may have equally important ecological roles to cleaner fishes (Becker and Grutter 2004).

Cleaner organisms are considered in the majority of the literature as either obligate or facultative. Youngbluth (1968) distinguished between obligate cleaners, those which rely almost exclusively on cleaning, and facultative cleaners which do not. This was based on Limbaugh's (1961) use of 'full-time' cleaners and reflected their diet and habits. Nevertheless,

there is no empirical evidence that any cleaner is truly obligate in the strict sense, as this would imply that these cleaning organisms would be compelled to derive all of their nutrition from their clients during such symbiotic interactions, without which they would perish. The definition of 'obligate' in a cleaning symbiosis is equivocal and this term should only be reserved for certain modes of parasitic or other symbioses where it holds true. The term 'dedicated' is proposed to replace 'obligate' when describing those cleaners that exhibit a committed mode of cleaning lifestyle throughout their non-larval ontogeny, and differentiate these from the other varying levels of facultative cleaners, those which are opportunistic, temporary cleaners or interact as cleaners only in part of their ontogeny. The consideration of Limbaugh (1961), that dedicated cleaners are more highly evolved than those that exhibit an opportunistic mode of cleaning, is difficult to evaluate, and may not necessarily be correct. Limbaugh (1961) considered that dedicated cleaners evolved from forms that were more free-living and exhibited opportunistic cleaning, while Gorlick *et al.* (1978) considered that at least members of one genus of dedicated cleaner fishes, *Labroides* Bleeker, 1851 may have evolved from an ectoparasitic form. However, Baeza (2009) concluded that, at least for some shrimp, the ancestral lifestyle was likely to have been equally symbiotic or free-living. A simpler explanation may be that animals that evolved to browse on epifauna would also browse on the skin of larger animals, be they mammals, turtles or large fishes. Cleaner fishes and shrimp obtain their food from cleaning and from the wider environment. The relative importance of each source is likely to vary in space and time, depending on client availability and parasite burden, cleaner appetite, and perhaps other factors.

Cleaning symbiosis was previously separated into two distinct categories; those examples which reflected traits that may have evolved to support cleaning, and those which reflected incidental cleaning. Côté (2000) considered incidental cleaning between organisms, under cleaning symbiosis, to include the removal and consumption of epibionts and debris

lodged on the body surface of one organism, by others as they might from any other suitable substrate. This category of cleaning symbiosis was not considered for further discussion in the review of Côté (2000) because neither 'cleaner' nor 'client' reflected any particular adaptation towards their respective roles (Côté 2000). The 'clients' and 'cleaners' from incidental cleaning interactions may both benefit from these interactions. However, incidental cleaning cannot be considered as cleaning symbiosis. Cleaning symbiosis is defined by the communication to clean or to be cleaned, either through assertion, or submission, resulting in cleaning through mutual cooperation. Assertion is the act of seeking out the cleaning interaction, either by the client or the cleaner, and is followed by the submission of the cleaner to clean, or the client to be cleaned. There is no apparent communication in incidental cleaning, which represents opportunistic mutualism. It may also be possible that all forms of communication that precede cleaning symbiosis have not yet been identified. In particular, none of the current cleaning symbiosis literature has explored the possible role of semiochemicals, or semiochemic communication (Regnier 1971) in cleaner-client signalling.

Interspecific semiochemicals are classifies into allomones, kairomones, and synomones (Brown *et al*. 1970; Nordlund and Lewis 1976), of which the latter includes those which benefit both interacting organisms. Synomones have been investigated previously in marine fishes, and have a direct effect on animal behaviour (e.g. Arvedlund et al. 1999; Karplus 2014). Chemoreception is considered to be one of the most important sensory modes in crustaceans (Breithaupt and Thiel 2011). It may be possible that symbiotic cleaning interactions are also influenced by these chemicals. This remains an important future focus of research.

Recent publications on marine turtles suggest that their epibiont burdens are a proximate cause of cleaning interactions with both fishes and shrimp (Losey *et al.* 1994; Sazima *et al*. 2004a; 2010), much like wounds and parasites on fishes are also a proximate cause of cleaning (Foster 1985; Arnal and Morand 2001; Grutter 2001; Sikkel *et al.* 2004;

Bertoncini *et al.* 2009). Such turtles actively seek out cleaners, and submit to them, to have their epibiont burdens removed, illustrating the importance of communication between client and cleaner to cooperate in a cleaning symbiosis. All true cleaning symbiosis interactions are preceded by some level of communication through assertion or submission, either by client or cleaner or both (examples discussed by Limbaugh 1961; Tyler 1963; McCutcheon and McCutcheon 1964; Feder 1966; Youngbluth 1968; Abel 1971; Able 1976; Ayling and Grace 1971; Hobson 1971, 1976; Losey 1972, 1974, 1979; Wyman and Ward 1972; Sargent and Wagenbach 1975; Sulak 1975; Brockmann and Hailman 1976; Corredor 1978; Minshull 1985; Sikkel 1986; Stauffer 1991; Soto *et al.* 1994; Van Tassell *et al.* 1994; Galeote and Otero 1998; Wicksten 1995, 1998; Poulin and Grutter 1996; Sazima *et al.* 1998b, Sazima *et al.* 2005; Côté 2000; Shigeta *et al.* 2001; Sazima and Moura 2000; Sazima and Sazima 2000; Becker *et al.* 2005; Shepherd *et al*. 2005; Craig 2007; Bertoncini *et al.* 2009; Horton 2011; Abe *et al.* 2012; Huebner and Chadwick 2012a; Karplus 2014). Dedicated cleaners and facultative cleaners actively assert their intentions to clean often by using conspicuous dances, or through tactile stimulation. Clients often pose submissively to signal a desire to be cleaned. Communication to cooperate is clearly the catalyst for cleaning interactions that not only transcends species boundaries in the same environment, but has also recently been shown to occur between the ocean sunfish (*Mola mola* (Linnaeus, 1758)) and Laysan albatrosses (*Phoebastria immutabilis* Rothschild, 1893) (see Abe *et al.* 2012). However, cleaning behaviour is not restricted to interspecific interactions, and has also been reported between members of the same species (Gooding 1964; Abel 1971; Able 1976; Hobson 1971, 1976; Sulak 1975; McCourt and Thomson 1984; Sikkel 1986; Soto *et al.* 1994; Shepherd *et al*. 2005; Krajewski 2007; Bertoncini *et al*. 2009; *cf*. Poulin and Vickery 1995).

Survival is difficult to quantify, but has an important effect on symbioses (Dickman 1992). However, where some symbioses may positively influence the survival of one symbiont

(e.g. parasitism), mutualisms, such as cleaning symbiosis, influence the survival of both symbionts positively. To highlight the importance of communication that results in cooperation between client and cleaner, an amended definition of cleaning symbiosis is proposed:

Cleaning symbiosis is a cooperative interspecific behaviour where a cleaner removes and consumes materials that negatively impact a client and is preceded by their communication.

Tactile stimulation in cleaning by fishes is considered an important influence on the initiation of cleaning (Losey and Margules 1974; Losey 1979), but may also be used to manage potential aggression shown by the client towards the cleaner (Grutter 2004), and may be a simple way of confirming that the cleaner is not a prey item because prey items are not likely to engage in direct contact with their predators. Wicksten (2009) questioned whether the association between examples of gregarious cleaner shrimp (*Lysmata* spp.) and morays reflected a cleaning symbiosis. However, subtle tactile stimulation with antennae and legs is offered by these shrimp prior to cleaning interactions (Chapuis and Bshary 2009). Furthermore, morays cooperate by opening their mouths in submission to these shrimp, communicating their acceptance to be cleaned (Limbaugh *et al.* 1961). Morays have poor eyesight and are nocturnal (Riordan *et al.* 2004). Therefore, visually-based communication by cleaners probably has less significance to morays than tactile stimuli. Indeed, tactile stimuli are considered significantly important for initiating cleaning interactions in fishes by cleaner shrimp and do elicit submissive client posture (Karplus 2014). Client fishes have been observed responding to these tactile stimuli at night, while relying more on sight during the day (Corredor 1978). In addition, morays are not known to actively seek out cleaning stations and may therefore rely more specifically on these facultative cleaners which co-habit their caves (Quimbayo *et al*. 2012).

Morays are also not the only clients that are known to be cleaned by these shrimp (Jonasson 1987; McCourt and Thomson 1984; Côté 2000; Wicksten 2009).

Additional anecdotal observations by SCUBA divers further add support that communication is the catalyst for cooperation in a cleaning symbiosis. Several images of diver-solicited cleaning responses of both fishes and shrimp to hands, feet and even teeth have been documented in the popular and social media (DBV personal observations), and in some of the scientific literature (Limbaugh *et al*. 1961; Brockmann and Hailman 1976; Kulbicki and Arnal 1999). Communication also appears to be important when ending a cleaning interaction, where clients twitch to indicate their desire to break the interaction, or they may also simply depart by swimming away (Feder 1966; Losey 1979; Poulin and Grutter 1996; Wicksten 1998; Wicksten 2009).

Familiar examples of marine cleaning symbioses are the most conspicuous, and usually involve dedicated cleaners, e.g. the bluestreak cleaner wrasse (*Labroides dimidiatus* (Valenciennes, 1839)) (see Bshary 2003), Hawaiian cleaner wrasse (*Labroides phthirophagus* Randall, 1958) (see Youngbluth 1968), the skunk cleaner shrimp (*Lysmata amboinensis* (de Man, 1888)) (see Chen and Huang 2012) and *Urocaridella* Borradaile, 1915 sp. c, Palaemonidae (see Becker *et al*. 2005). These cleaners are often synonymous with cleaning stations located at strategic points on the reef, and have been relatively well studied. Facultative cleaner fishes have been comparatively underinvestigated, but may forage more widely than dedicated cleaners. There appears to be a greater diversity of facultative cleaner species than dedicated cleaners (Côté 2000; Tables 2.1, 2.2). However, comparatively little work has been done to evaluate differences in client diversity between dedicated and facultative cleaners. Some cleaners are adapted to live closely with their clients. These include some members of the Echeneidae (Cressey and Lachner 1970) and Alpheidae (Karplus *et al.* 1972; Hou *et al.* 2013) which interact with their clients as true commensals (Strasburg 1959) as well as cleaners.

Some dedicated cleaner shrimp are also known to associate with anemones, which they use for shelter and protection but also to signal the locations of their cleaning stations to client fishes (Huebner and Chadwick 2012b).

2.4 Cheating

Cleaners have been reported to remove and ingest client fish mucus and scales in addition to their ectoparasites; clients have been reported to eat their cleaners. Both are classic examples of cheating in a cleaning symbiosis (Randall 1958; Limbaugh *et al*. 1961; Feder 1966; Hobson 1971; Gorlick 1980; Grutter 1997; Francini-Filho *et al*. 2000; Arnal *et al.* 2001; Grutter and Bshary 2003; Cheney and Côté 2005; Soares *et al.* 2008a; Oates *et al*. 2010). Cheating is a temporary disturbance in the symbiotic relationship (Bshary and Würth 2001), not isolated to cleaning symbiosis, but is common in many mutualisms, and results when one partner provides less commodity for their benefit received (Ferreire *et al.* 2001). Several studies conducted on cleaner fishes have indicated that fish mucus is a potentially valuable and more reliable source of food for the cleaner than ectoparasites whose abundance may vary seasonally, between localities, and client species (Gorlick 1980; Youngbluth 1968; Grutter 1997; Arnal *et al*. 2001). This may tempt the cleaner to cheat by taking mucus and scales instead of ectoparasites when afforded the opportunity. In the cleaner wrasse *L. dimidiatus*, individuals of a male and female pair cleaning together reduce each other's cheating when working together (Bshary *et al.* 2008). However, when they operate individually, they show a higher rate of cheating in both males and females (Bshary *et al*. 2008). Client fishes often respond to cheating by terminating the interaction by swimming away, or by chasing the cleaner in what has been considered as cleaner punishment (Bshary and Grutter 2002; 2005). Client fishes without the option of moving away (e.g. in captivity) generally react more aggressively to cheating (Bshary and Grutter 2002). Client fishes that may not have been directly involved in a cheating event may

also show reluctance to be cleaned by a cheating cleaner. Client fishes may exhibit an image-scoring strategy which involves bystander clients observing the quality of cleaning offered by the cleaner to other clients (Bshary 2002; Bshary and Grutter 2006). Through observation of cleaning behaviour, client fishes may then show a preference to interact with cleaners that show a lower tendency to cheat (Bshary 2002).

The majority of reports on cheating in marine cleaning symbioses deal with cleaners as the cheater, and few comparisons have been made of the frequency of cheating by dedicated versus facultative cleaners. Cheating is generally considered supportive of the biological market hypothesis, where cheating by cleaners is proportional to the number of clients available to cleaners (Akçay 2015). However, facultative cleaners probably have less to lose from dishonest interactions than dedicated cleaners, but recent evidence suggests that some facultative cleaner fishes (wrasses) cheat less than dedicated cleaner fishes. This is thought to result from them not feeding against their food preference of client ectoparasites (Barbu *et al*. 2011; Gingins and Bshary 2016), unlike the dedicated *L. dimidiatus* which is known to prefer host mucus under certain conditions (Bshary and Grutter 2005, 2006).

Cleaner shrimp have been shown to adjust their cleaning strategy to the clients they serve and the risk of predation (Chapuis and Bshary 2009; Huebner and Chadwick 2012a). Cheating by the long-arm cleaner shrimp (*Ancylomenes longicarpus* (Bruce & Svoboda, 1983)) produced similar client responses as cheating cleaner wrasse (*L. dimidiatus*), and less reaction from predatory species than from non-predatory species (Chapuis and Bshary 2009). This suggested that the shrimp can distinguish between these types of clients. The observed variability in cleaning behaviour in Perderson's shrimp (*Ancylomenes pedersoni* (Chace, 1958)) may be controlled, to some extent, by some client fishes that interfere with access to the shrimp by other clients (Huebner and Chadwick 2012a). However, these shrimp may also influence each other's cheating during cooperative cleaning interactions as cleaner wrasse do

(Huebner and Chadwick 2012a). It thus appears that both cleaner fishes and shrimp can discern different types of clients and therefore the risk they take if they cheat.

Historically, cheating was thought to inhibit mutualism, resulting in 'reciprocal extinction' (Roberts and Sherratt 1998; Doebeli and Knowlton 1998). However, Ferreire *et al*. (2001) proposed that cheating can establish a foundation to support competitively superior mutualists which may result in the evolution of different related and unrelated cheater and mutualist phenotypes and their coexistence.

2.5 How many cleaners are there?

Over the last half century, the number of fishes and crustaceans considered as cleaners has increased significantly, demonstrating the development of our understanding of cleaning symbiosis (Fig. 2.2). Here, the extensive primary literature to date was reviewed and cross-referenced, and a current list of marine and freshwater fishes and marine crustaceans populated which includes a number of species either missed by previous workers, or species for which evidence of cleaning has been published since the last reviews of Côté (2000) and Karplus (2014). In addition, the list also includes the juvenile sunburst butterflyfish (*Chaetodon kleinii* Bloch, 1790) observed and photographed by one of us (DBV) for the first time cleaning the brownburnie (*Chaetodon blackburnii* Desjardins, 1836) with a confirmed infection of the parasitic dinoflagellate *Amyloodinium ocellatum* (E. Brown) E. Brown & Hovasse, 1946 in captivity (Fig. 2.3). Observations of cleaning symbiosis in captivity were excluded by Côté (2000), but these are included here because it cannot be assumed that captivity produces only artificial behaviour, and well-known cleaner organisms of various species observed cleaning in the wild are also observed to exhibit the same cleaning behaviour in captivity, and are exploited in home and public aquaria, and in aquaculture for this reason. There are currently approximately 208 species of cleaner fishes from 106 genera representing 36 families and 51

species of cleaner shrimp from 11 genera representing six families, recorded to exhibit cleaning behaviour (See Tables 2.1, 2.2; Fig. 2.4). Although *Urocaridella* sp. a, b and c are discussed in this review as examples of cleaner shrimp in the literature, these shrimp are not listed in the supplemental information because they remain currently undescribed. Both Tables 2.1 and 2.2 consider only valid described taxa and are updated to the current relevant taxonomy. Synonyms are included in the footnotes of both Tables 2.1 and 2.2. Reports of other putative cleaners (see footnotes of Tables 2.1, 2.2) are excluded for a lack of supporting evidence or verifiable source, or because their taxonomic identity could not be confirmed, or due to their original listing in error by other authors. Observations of cleaning interactions by fishes and shrimp span the Americas, Europe, Africa, Asia and Oceania (Figs. 2.5, 2.6). They include freshwater and marine environments for fishes. However, they have only been reported for less than half of likely countries for fish (Fig. 2.5) and less again for shrimp (Fig. 2.6). Thus cleaning behaviour is geographically widespread and likely to be more ecologically significant than the present limited observations indicate.

2.6 Consider the grey literature with caution

The grey literature and the correspondence of divers are both difficult to assess for accuracy. Becker and Grutter (2004) reviewed the scientific, marine, SCUBA and aquarium hobbyist guides to produce more than 40 species records of cleaner shrimp and this estimate has been generally accepted in the field (McCammon *et al.* 2010; Hou *et al.* 2013). Although observations should not be discounted as empirical evidence, they do require verification. The identification of many cleaner fishes and shrimp is not simple and many cleaners have been confused, misidentified, and/or form part of a species complex (see footnotes of Tables 2.1, 2.2). This suggests that misidentification of species, resulting from the lack of proper taxonomic verification, may significantly influence the bias of data from grey literature or

observer accounts of cleaning interactions. Therefore, these accounts should be carefully evaluated before being incorporated into scientific literature.

Spotte (1998) had a more cautionary view and dismissed the contributions of all observations on cleaner shrimp in the historic literature as anecdotal, with the exception of Turnbull's (1981) unpublished PhD thesis which Spotte (1998) considered the only work to properly assess a shrimp cleaning symbiosis at that time. Turnbull (1981) found no remnants of ectoparasites in the foregut of *Ancylomenes pedersoni*, nor did he observe the removal of conspicuous crustacean ectoparasites from client skin surfaces by *A. pedersoni*. In conclusion, Turnbull (1981) stated that *A. pedersoni* did not possess the functional morphology to confirm this shrimp was a cleaner (Limbaugh 1961). However, his observations using SCUBA were undoubtedly of larger adult stages of parasitic crustaceans, as these were visible, and the midgut section of the shrimp may have revealed remnants of ectoparasites (Tziouveli *et al.* 2011). Although Spotte (1998) considered this evidence enough to suggest that cleaner shrimp as cleaners of fishes be dismissed, Bunkley-Williams and Williams (1998) and McCammon *et al.* (2010) provided empirical evidence to the contrary for the same species in a laboratory trial and semi-natural exhibit system, respectively. The study of Bunkley-Williams and Williams (1998) was the first laboratory study to provide such evidence in support of cleaning by a shrimp species. Their results also suggested that cleaner shrimp may be specialists rather than generalists because only one of the four cleaner shrimp species tested removed and consumed juveniles of the parasitic cymothoid isopod *Anilocra haemuli* Williams and Williams, 1981.

If the view of Spotte (1998) was considered to the exclusion of all observations of cleaning interactions in the literature, there would only be six shrimp considered as cleaners, notably *Ancylomenes holthuisi* (Bruce, 1969) and *Urocaridella* sp. c. (see Becker and Grutter 2004), *A. pedersoni* (see Bunkley-Williams and Williams 1998; McCammon *et al.* 2010), *Lysmata amboinensis* (see Militz and Hutson 2015), and *Palaemon adspersus* Rathke, 1837

and *Palaemon elegans* Rathke, 1837 (see Östlund-Nilsson *et al.* 2005). The view of Spotte (1998) is probably premature. The mechanisms involving costs and benefits of cleaning symbiosis are not yet fully understood (Cushman and Beattie 1991; Poulin and Vickery 1995; Cheney and Côté 2003; Orr 2009), and recent evidence suggests these costs and benefits extend beyond the traditionally defined symbiotic interaction to secondary benefits, including the reduction of ectoparasites in the environment (Bshary 2003; Grutter *et al.* 2003; Waldie *et al.* 2011; Militz and Hutson 2015).

2.7 Literary ambiguities and inconsistencies

Cleaner shrimp are only known from the marine environment. The colloquial term 'cleaner shrimp' was used broadly by Davie (2002) for all members of the Infraorder Stenopodidea, and by Wicksten (1995) to refer to the shrimp families Stenopodidae, Palaemonidae, and Hippolytidae. However, not all genera and species representing these families have been observed to form cleaning symbioses (Bruce and Baba 1973, Bruce 2004, and Baeza 2010, respectively). Debelius (1999) used the same colloquial term for all *Lysmata* species, and also mentioned that all species of *Stenopus* were 'probably' cleaners. However, the original description of *Stenopus chrysexanthus* Goy, 1992 and redescription of *Stenopus cyanoscelis* Goy, 1984 only assumed that both these species *may* be cleaner shrimp. This assumption was based on their similar morphology with other species known to engage in cleaning symbiosis, but it was not supported by observations or additional data on recorded symbiotic interactions. These species were therefore not included in the comprehensive review on cleaner fishes and crustaceans by Côté (2000), and remain excluded here. Subsequently, Poore (2004) introduced species of *Stenopus* as 'fish cleaners', and in a later publication, Goy (2010) made the explicit statement that all members of *Stenopus* enter into mutualistic cleaning symbiosis with coral reef fishes, citing Limbaugh *et al*. (1961), Yaldwyn (1968), Criales and Corredor (1977),

Jonasson (1987), Wicksten (1995, 1998), Côté (2000), and Becker and Grutter (2004). However, none of these authors that Goy cited dealt with the genus *Stenopus* in its entirety; they only referred to *Stenopus hispidus* (Olivier, 1811) and/or *Stenopus scutellatus* Rankin, 1898 (see Limbaugh *et al*. 1961; Criales and Corredor 1977; Jonasson 1987; Wicksten 1995, 1998; Côté 2000), or *S. hispidus* and *Stenopus tenuirostris* de Man, 1888 (see Yaldwyn 1968) specifically, or included Stenopodidae with six other families from which cleaner shrimp have previously been recorded (Becker and Grutter 2004).

Three problems emerge from defining shrimp genera or families as 'cleaner shrimp'. Firstly, the colloquial term 'cleaner shrimp' is used ambiguously for taxa that are known to engage in cleaning symbioses and for related taxa that currently are not known to (e.g. Wicksten 1995; Debelius 1999; Davie 2002). This ambiguity has spilled over into scientific literature. Martinelli-Filho *et al*. (2008) recently presented the species *Periclimenes paivai* Chace, 1969, a commensal palaemonid of scyphozoan jellyfish, as 'cleaner shrimp'. Martinelli-Filho *et al*. (2008, page 134) stated that "the genus *Periclimenes* contains more than 175 species of small carideans, commonly known as cleaner shrimps." The genus *Periclimenes* Costa, 1844 was represented by 10 cleaner shrimp species prior to the transfer of most of these to the new genus *Ancylomenes* by Okuno and Bruce (2010). Currently, only one species of cleaner shrimp is representative of *Periclimenes*, *P. yucatanicus* (Ives, 1891). Second, shrimp species unconfirmed as cleaners are conferred 'cleaner' status by association with their close relatives for which there is empirical cleaning evidence. Examples of this include the introduction of *Stenopus* by Poore (2004) as 'fish cleaners', and the 'cleaner symbionts' of Davie (2002) for *S. chrysexanthus* and *S. cyanoscelis*, citing Goy (1992). Third, the cited historic literature by several authors does not support the claim that all *Stenopus* species enter into cleaning symbioses. The likely explanation for this is that the statements of Debelius (1999), Poore (2004), and Goy (2010) must reflect other legitimate field or laboratory

observations, but which have remained unpublished. Indeed, correspondence with one of these authors confirmed that this information originated from the combination of laboratory studies and correspondence from numerous SCUBA divers. The possible argument that the above claim is common knowledge is unfounded because there is no original verifiable source. Therefore the use of the term 'cleaner shrimp' is encouraged only for representing shrimp that have documented observations of cleaning behaviour.

2.8 Diet

There is no evidence to suggest that cleaner organisms will eat all perceivably diverse ectoparasites as might be inferred by the original definition of a cleaning symbiosis. Cleaners feed mainly on crustacean ectoparasites (Table 2.3), client skin and mucus. Members of the marine isopod family Gnathiidae feature as prey items of 22 cleaner species, representing 15 genera (Table 2.3), and may be the most common parasitic prey item available to cleaners (Rohde 2005). These isopods feed on their hosts as three juvenile unfed zuphea stages and take a blood meal before vacating the host to moult into the next juvenile stage or complete their life-cycle as non-feeding adults (Rohde 2005). The engorged "praniza" stages may present a particularly rich source of food for the cleaner, much like engorged ticks do for several birds observed in terrestrial cleaning interactions (Rohde 2005; Sazima *et al.* 2012). Although crustacean ectoparasites may appear from the literature to be superior prey items for cleaners, this may reflect sampling bias because only crustacean exoskeletons provide a reliable means of identification in morphological gut analyses (Kearn 1978). Additionally, several publications have excluded other parasite taxa from their analyses and focussed almost exclusively on crustaceans (Grutter 1997; Arnal and Côté 2000; Arnal and Morand 2001; Cheney and Côté 2001, 2005; Whiteman and Côté 2002). However, in laboratory experiments

the cleaner wrasse *L. dimidiatus* consumed more monogeneans than gnathiids when presented with a choice (Grutter and Bshary 2003).

Monogenean ectoparasites, leeches, and protists, unlike the crustaceans, are soft-bodied which presents a problem for their identification in gut analyses. Many of these ectoparasites that infest fishes are very small in comparison to the often larger and more visible crustacean ectoparasites. For example, most *Gyrodactylus* von Nordmann, 1832 spp. measure 0.4mm – 0.8mm (Kearn 1999) *versus* 1.1mm – 6.1mm for seven representative *Gnathia* Leach, 1814 spp. (see Diniz *et al*. 2008). Although many of the soft-bodied ectoparasites of fishes present no structures that remain intact after digestion that can be used for potential taxon identification, the majority of monogeneans do. Monogeneans attach to their host fishes using the posterior attachment organ, the haptor, which often contains sclerotised attachment anchors, hooks, clamps or other modified structures that are very small but resist the digestion by proteolytic enzymes (Vaughan and Chisholm 2010). It may be possible to discern these structures in the gut samples of cleaners under high magnification (e.g. Grutter 1997; Becker and Grutter 2004). Various universal primers have been designed for use in metagenomic profiling (Folmer *et al*. 1994; Blankenship and Yayanos 2005; King *et al.* 2008) and a highly sensitive molecular approach may be successful in providing some resolution on what different organisms are consumed by different cleaners in the wild. This has been achieved for free-living marine decapod larvae (O'Rorke *et al*. 2012; 2014).

Adult parasitic stages of some parasites may simply be too large for some cleaners to remove from the client, which might explain the differences in observations between studies on the same cleaner species (*cf*. Turnbull 1981; Bunkley-Williams and Williams 1998). Differences in cleaning performance, or feeding preferences are known in cleaner fishes (Costello 1996), and this may be true for cleaner shrimp. The differences in morphology between cleaner shrimp species may limit them to feeding on specific types or life-stages of

certain parasites, or may even limit them as wound cleaners. Indeed, Bunkley-Williams and Williams (1998) were unsure of the mechanism of juvenile *A. haemuli* removal employed by *Ancylomenes pedersoni* in their experiments, and no studies have been conducted to evaluate whether there is a relationship between the functional morphology and the types of parasites removed and cleaning performed. Some shrimp are well documented as dedicated fish cleaners and exhibit strong symbiotic associations with fishes, whereas others are opportunistic facultative cleaners that are also scavengers, or the cleaning association remains insufficiently known (Davie 2002; Table 2.2).

Juvenile ectoparasites may be an important food items for cleaner organisms. The study of Becker and Grutter (2004) was the first study to provide evidence of parasitic removal and consumption in wild cleaner shrimp. These cleaner shrimp, *A. holthuisi* and *Urocaridella* sp. c, consumed juvenile parasitic gnathiids and copepods that were identified to family and class respectively. No other work since Becker and Grutter (2004) has examined the gut contents of wild cleaner shrimp. However, both these shrimp species appeared to have different diet preferences and/or consumption rates of ectoparasites (Becker and Grutter 2004). Laboratory trials using *A. holthuisi* and *Urocaridella* sp. c (Becker and Grutter 2004), and *Palaemon adspersus* and *P. elegans* (Östlund-Nilsson *et al.* 2005) revealed that cleaner shrimp can also consume monogenean ectoparasites. Monogeneans have never been found in the gut contents of wild shrimp. However, Militz and Hutson (2015) indicated for the first time that the cleaner shrimp *L. amboinensis*, a dedicated cleaner, was highly efficient in consuming the monogenean eggs and free-swimming larvae of the monogenean *Neobenedenia* sp. in the captive environment, and thus reduced reinfection success.

Approximately 111 fish ectoparasite records exist from dietary constituents of 49 different cleaner fishes (Table 2.3), and have been confirmed through wild fishes' gut content analyses, or observed being removed by cleaner fishes in captivity. However, the potential

diversity of dietary components of cleaner shrimp remains uninvestigated. It is unknown whether cleaner shrimp consume other pathogenic agents, including other parasitic groups such as leeches and protists, bacteria and water moulds. Foster (1985) documented wound healing of injured reef fishes by three different cleaner fishes, and suggested that cleaner shrimp removal of necrotic or diseased tissue may also promote wound healing. Although some anecdotal information claims that cleaner shrimp remove or consume dead skin from wounds (Corredor 1978; Crump 2009), or tend bacterial infections (Limbaugh 1961), the effects of cleaner shrimp on wound healing also remains uninvestigated and controlled experiments are needed to accurately address these questions.

2.9 Morphology, colour and behaviour

Côté (2000) analysed body size and signalling colouration of cleaner fishes. Her analyses were limited due to a lack of phylogenetic information on fishes at that time, and the correlation between body size and adult feeding type. Subsequently, Baliga and Mehta (2015) determined the kinematic basis of cleaning in three cleaner fishes of the family Labridae, suggesting that a small mouth gape and the ability to perform rapid gape cycles (opening and closing of the mouth) on individual prey items may be a cleaner-prerequisite. Certainly, many juvenile fishes that are facultative cleaners have a small gape, which may support a rapid and dextrous ability to remove ectoparasites on clients (Baliga and Mehta 2015). Ontogenetic prey-use change is known in a large diversity of marine reef fishes (McCormick 1998; Wainwright and Bellwood 2002), and it is unsurprising, given the ubiquity of fish ectoparasites, that so many fishes utilise this resource during their ontogenetic development.

Cleaner shrimp vary considerably in size between species and genera. Their size may influence the ability to remove and consume certain ectoparasites, for which they use their chelae (Yaldwyn 1968; Östlund-Nilsson *et al*. 2005; Karplus 2014), but small size also

facilitates access into areas of the mouth and gill chamber of client fishes (Karplus 2014). An increase in the robustness of the mandibles, as well as the morphological intricacy of the gastric mill reflects a carnivorous feeding habit in crustaceans (Kunze and Anderson 1979). Conversely, the paragnaths in carnivorous crustaceans are less intricate than those of non-carnivores (Hunt *et al.* 1992). The investigation of the comparative morphology of these structures between different cleaning shrimp may help determine what these shrimp consume in the wild (Tziouveli *et al*. 2011).

The concept of a universal colour guild for cleaners was not conclusively supported by the analyses of Côté (2000), and whether cleaners use colour to signal cleaning services remains untested. Although longitudinal striping is a common feature of dedicated cleaner fishes (Côté 2000) and is now demonstrated for a facultative cleaner (see Carvalho *et al*. 2003), all considerations of cleaner colouration or patterning made to date have been limited to the visible light spectrum. Ultraviolet light has a fundamental function in the mutualism between angiosperms and their pollinators (Papiorek *et al*. 2016), and ultraviolet reflective body patterns have been demonstrated as a means of communication in fishes that can visualise ultraviolet (Siebeck *et al*. 2010). Therefore, ultraviolet patterning may be important for cleaner recognition, and suggest that future investigations should include ultraviolet patterning of cleaner organisms.

Cleaner shrimp vision is likely monochromatic. Recent work investigated the visual ability of *A. pedersoni*, *L. amboinensis*, and *Urocaridella antonbruunii* (Bruce, 1967) for the first time (Caves *et al*. 2016). The spatial resolution of these shrimp, and possibly others, is less than for sea snails and scallops, and decreases with a decrease in light (Caves *et al*. 2016). This research suggests that cleaner shrimp cannot assess client fish for ectoparasites visually, as suggested in part by Becker and Grutter (2005), and that tactile and chemical stimuli are used to detect ectoparasites on client fishes. The colour limitation of cleaner shrimp vision also

suggests that the change in client pigmentation often seen during cleaning may be a visual signal to other client fishes, rather than the cleaner (Caves *et al*. 2016).

Becker and Grutter (2005) provided evidence that ectoparasite load and cleaner shrimp hunger levels influence cleaning interactions. Apart from these factors, very little information is available on what drives the processes behind the cleaner shrimp-client interactions (Titus *et al.* 2015). However, recent evidence suggested that temporal patterns of cleaning between *A. pedersoni* and cleaner gobies differed, but the client species and localities were the same. Titus *et al.* (2015) considered that the ectoparasites targeted by the shrimp may be different to those targeted by the cleaner gobies, which would explain the apparent lack of competition for the same clients. In addition, there are no data to compare the difference in cleaning quality between cleaner shrimp species.

2.10 The ecological importance of cleaning symbioses on coral reefs

Cleaner organisms maintain an ecological balance that is not yet fully understood, although it is clear that the removal of ectoparasites is beneficial for the health of reef fishes. Several authors have attempted to quantify the effects of cleaner fishes on reef fish diversity by testing the hypothesis that the removal of cleaners presents a perturbation of the ecosystem, resulting in reef fishes' emigration, or mitigation by remaining and/or unfamiliar cleaners (Losey 1972). Limbaugh (1961) was the first to present observations on the possible effects of cleaner removal from a reef. He removed all known cleaner organisms from two isolated parts of Bahamian reef containing a high diversity of fishes. This resulted in a considerable reduction in the number of fishes observed, as well as the observed increase in visible lesions on remaining territorial fishes (Limbaugh 1961). Presumably, these lesions resulted from the absence of cleaners.

In a similar *L. phthirophagus* depopulation experiment off Hawaii, Youngbluth (1968) did not observe a significant decrease in the number of fishes after the removal of cleaners. In comparison, Youngbluth (1968) considered the possibility that differences in the physical properties of the reefs in both studies may have influenced the movement of fishes to different areas. Gorlick *et al.* (1978) were highly critical of Limbaugh (1961), and in a subsequent cleaner wrasse (*L. dimidiatus*) depopulation study off the Marshall Islands (see Gorlick *et al*. 1987), these authors found no significant change in the density of fishes before and after cleaner removal. However, Losey (1972) removed all *L. phthirophagus* from patches of reef in Hawaii and found that there was a change in the behaviour in some client species that relocated to patches of reef with a remaining *L. phthirophagus*, and some facultative cleaners that increased their cleaning activity to some degree. Losey (1972) did not find a significant reduction in ectoparasites after the removal of *L. phthirophagus*, which was in contrast with the suggestion of Limbaugh (1961) that "cleaners maintain the health of the marine population," and that of Gorlick *et al.* (1987) who determined that *L. dimidiatus* reduced ectoparasite biomass. Variation in the importance of cleaner fishes and shrimp is to be expected. Host abundance, parasite burdens and pathogenicity, and cleaner abundance and appetite will vary in space and time. Further research is required to clarify the importance of cleaners in food webs and ecosystems through their effects on client health.

The role of time in symbiotic relationships is important in determining functional outcomes and avoiding their misinterpretations. The balance between costs and benefits may change with time, which in turn may influence these functional outcomes (Mesterton-Gibbons and Dugatkin 1992, 1997). Limbaugh's (1961) observations were for a period of two weeks, while the studies of Youngbluth (1968) and Gorlick *et al*. (1987) were concluded after one and six months, respectively. Losey's (1972) cleaner removal experiment was for eight months. Bshary (2003) considered the removal of *L. dimidiatus* for less than four months to be short-

term, with subsequently few observed effects on fish diversity. However, a significant decline in reef fish diversity was evident over a longer period of up to twenty months (Bshary 2003). Conversely, the introduction of an additional cleaner wrasse, or the relocation of one to a patch of reef previously without one, influenced a rapid increase in fish diversity (Bshary 2003). This suggested that the studies of Limbaugh (1961) and Losey (1972) reflected a rare effect, or that the studies of Youngbluth (1968) and Gorlick *et al*. (1987) were too short to identify a significant ultimate outcome.

Longer-term studies on the ecological influence of cleaners have revealed limitations in short-term studies. Grutter *et al.* (2003) and Waldie *et al.* (2011) found evidence of a decrease in general fish diversity and abundance after the experimental removal of *L. dimidiatus* from patches of reef off Lizard Island, Australia. Grutter *et al*. (2003) noted a reduction in transient fishes after 18 months, and Waldie *et al*. (2011) noted the reduction for both transient and territorial fishes over an eight and a half year period with the removal of *L. dimidiatus*. The reduction in territorial species including pomacentrids and the shift towards smaller individuals in two pomacentrids in the study by Waldie *et al.* (2011) was considered the result of lower growth rates and/or the reduced survivorship of these species in the absence of cleaner wrasse. The length of the study also demonstrated the influence of cleaner wrasse on the recruitment of the juveniles of transient fishes onto the reef (Waldie *et al*. 2011) as did an even longer 12 year study involving juveniles of territorial fish (Sun *et al.* 2015). The consideration of transient and territorial fishes in these studies plays a subtle yet important role. Grutter *et al.* (2003) were the first authors to suggest the importance of distinguishing between these types of fishes in these types of studies. Pomacentrids for example, and particularly the monodomous species (Fishelson 1998), can confound such results of reef species movement because of their strict territorial habits (Bardach 1958). Pomacentrids are more likely to remain in their territories after cleaner organism removal, as shown by Grutter (1996a) for the lemon

damselfish (*Pomacentrus moluccensis* Bleeker, 1853) observed in a previous depopulation study on cleaner wrasse (*L. dimidiatus*). Similarly, Bshary (2003) showed that the presence or absence of cleaner wrasse (*L. dimidiatus*) had the weakest effect on territorial species. However, neither Youngbluth (1968) nor Gorlick *et al.* (1987) made the distinction between transient and territorial fishes in their studies. Gorlick *et al*. (1987) specifically included the territorial ocellate damselfish (*Pomacentrus vaiuli* Jordan & Seale, 1906) in their study, but did not list the other client species involved in the depopulation study, and it is unclear what influence this and possibly other territorial species could have had on their results.

No comparative depopulation studies have been conducted for cleaner shrimp, although this would also prove to be extremely difficult because cleaner shrimp are cryptic and physically delicate. In addition, many species of shrimp may currently be unknown cleaners, similar to the growing list of fish cleaners that has developed over the past 50 years (see Fig. 2). However, this does pose the question of the involvement of cleaner shrimp in the above-mentioned cleaner fish depopulation studies. One unidentified shrimp was observed by Losey (1972) cleaning the millet butterflyfish (*Chaetodon miliaris* Quoy & Gaimard, 1825), but Gorlick *et al*. (1987) did not observe any cleaner shrimp. Whether this reflects sampling and observation bias, or an extended observation of 'cleaning structure discordance' between fishes and shrimp as mentioned by Titus *et al.* (2015), remains to be elucidated.

2.11 Exploitation of cleaning in captivity

The published observations of Potts (1973) may have inspired the first investigations using cleaner fishes as alternative methods of ectoparasite control in aquaculture. Caligid copepod sea lice are the most persistent and economically significant parasite in marine salmonid farming worldwide (Costello 2006, 2009). Following reports from fish farmers using cleaner fishes (Labridae) to control lice on salmon in farm cages in Norway, experiments in Ireland

and Scotland showed that five common labrids in northern Europe could reduce lice abundance on farmed salmon to non-pathogenic levels within weeks (Costello 1993a; 1996), namely Rock cook (*Centrolabrus exoletus* (Linnaeus, 1758)), goldsinny (*Ctenolabrus rupestris* (Linnaeus, 1758)), Corkwing (*Symphodus melops* (Linnaeus, 1758)), cuckoo wrasse (*Labrus mixtus* Linnaeus, 1758) and juvenile ballan wrasse (*Labrus bergylta* Ascanius, 1767). Now several million of these cleaner fishes are routinely used in Norway, mostly wild captured (Bjordal 1991; Darwall *et al*. 1992; Skiftesvik *et al*. 2014). Initially it was believed that only juvenile *L. bergylta* showed cleaning behaviour (Costello 1993b), but it has since been shown that adults will clean larger salmon (Skiftesvik *et al.* 2013). Research into culturing certified disease free labrids to supply the farms is also underway (e.g. Skiftesvik *et al*. 2013). In addition, lumpfish (*Cyclopterus lumpus* Linnaeus, 1758) are being developed for use as cleaner fish on farms (Imsland *et al*. 2014a). The use of cleaner fishes reduces or avoids the need to use parasiticides to control lice, thereby improving fish health, saving costs, and the farmed fish can be harvested without drug residues. Options for lice control are constrained because lice have developed resistance to all the parasiticides used on the farms to date (Costello *et al.* 2001; Costello 2006; Aaen *et al.* 2015). The main limitations to using cleaner fishes have been adequate supply, their ability to escape, and the influence of environmental conditions on cleaning activity and ectoparasite growth rates (Costello 2006). Recent concerns suggest that wrasse species used as cleaners in Europe may also be the reservoirs of diseases in Atlantic salmon culture, for example viral haemorrhagic septicaemia (Munro *et al.* 2015; Wallace *et al.* 2015), amoebic gill disease (Karlsbakk *et al.* 2013), and *Aeromonas salmonicida* (Aeromonadaceae) (Treasurer 2012), further supporting certification of disease-free cultured cleaners.

There have been no observations of either client (salmonid) or cleaner (labrid or lumpfish) communication to cooperate prior to cleaning interactions in the farms or laboratory (e.g. Imsland *et al*. 2014a, b). However, the wrasse species do hover above the seabed in the

wild and clean fishes that remain stationary in their territory (Costello 1993b, MJC personal observations). It is possible that this communication has been overlooked in captivity, or that the cleaning interactions in intensive cage culture simply reflect incidental cleaning (opportunistic mutualism) and not true cleaning symbiosis.

In tropical aquaculture the cleaner gobies of the genus *Elacatinus* have been investigated for their potential as biological controls against ectoparasites, particularly against monogeneans. *Elacatinus genie* (Böhlke and Robins, 1968) and *Elacatinus oceanops* Jordan, 1904 have shown promise against the problematic monogenean *Neobenedenia melleni* (MacCallum, 1927) on cultured euryhaline tilapias (Cowell *et al*. 1993), and *Elacatinus figaro* Sazima, Moura and Rosa, 1997 was recently tested successfully for its efficacy against *N. melleni* on the aquaculture candidate species *Epinephelus marginatus* (Lowe, 1834) in Brazil (de Souza *et al*. 2014). *Elacatinus oceanops* has also been used successfully with cultured mutton snapper (*Lutjanus analis* (Cuvier, 1828)) and greater amberjack (*Seriola dumerili* (Risso, 1810)) (see Benetti *et al.* 2007; de Souza *et al.* 2014), and cobia (*Rachycentron canadum* (Linnaeus, 1766)) broodstock (Benetti *et al*. 2007). Tropical cleaner wrasse species have not yet been considered for aquaculture. *Labroides dimidiatus* is, however, used as a biological control against ectoparasites in public aquaria (Paul Lötter pers. comm.), and cleaner fish were suggested as a biological control for the ectoparasites of captive rays by Chisholm *et al.* (2004).

Cleaner shrimp have not been used as biological controls in aquaculture. However, Becker and Grutter (2004) and Militz and Hutson (2015) suggested their potential benefits for ectoparasite control in aquaculture. One of the advantages of cleaner shrimp over cleaner fishes in aquaculture is their unlikely function as disease reservoirs or vectors compared with cleaner fishes (Militz and Hutson 2015), given the paucity of reports of diseases affecting shrimp being transmitted to fishes. Cleaner shrimp also actively consume environmental parasite stages such

as monogenean eggs and larvae (Militz and Hutson 2015) which implies their usefulness as direct and indirect cleaners. They could be integrated into sections of the aquaculture system itself, away from client fishes, particularly in recirculating systems. There may also be value in the integration of both cleaner wrasse and shrimp in combination in aquaculture.

It has been documented that some client fishes change colour during posturing; its reason is unclear. Future research priorities should include the investigation of possible cleaner and client recognition by ultraviolet reflective patterning, and whether client posturing may enhance their visibility and/or that of their ectoparasites. Indeed, communication by other sensory mechanisms also require study. Additionally, understanding the ecological role of cleaner shrimp can be advanced using a combined morphological and molecular investigation of gut contents to elucidate the diversity of prey items consumed.

Acknowledgements

I wish to acknowledge the support of, and comments on the manuscript by Howard Feder (retired), to whom the published article is dedicated. I thank Martin Gomon (Museum Victoria, Australia) for checking the fishes' taxonomy.

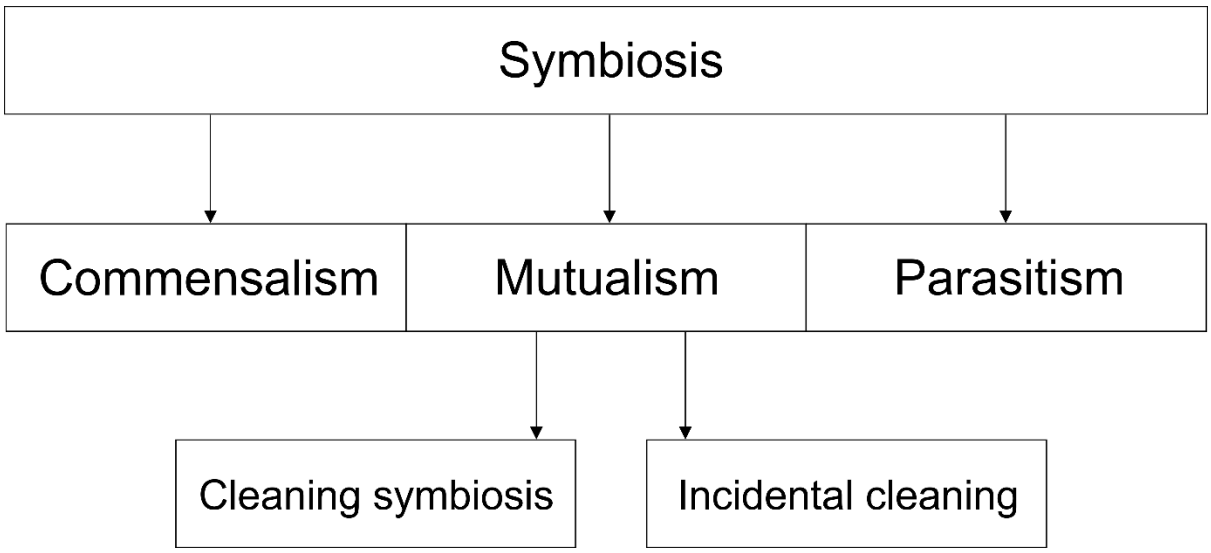

Fig. 2.1. Symbiosis is the collective term for commensal, mutual and parasitic associations between organisms. Cleaning symbiosis and incidental cleaning are considered mutualistic associations under symbiosis.

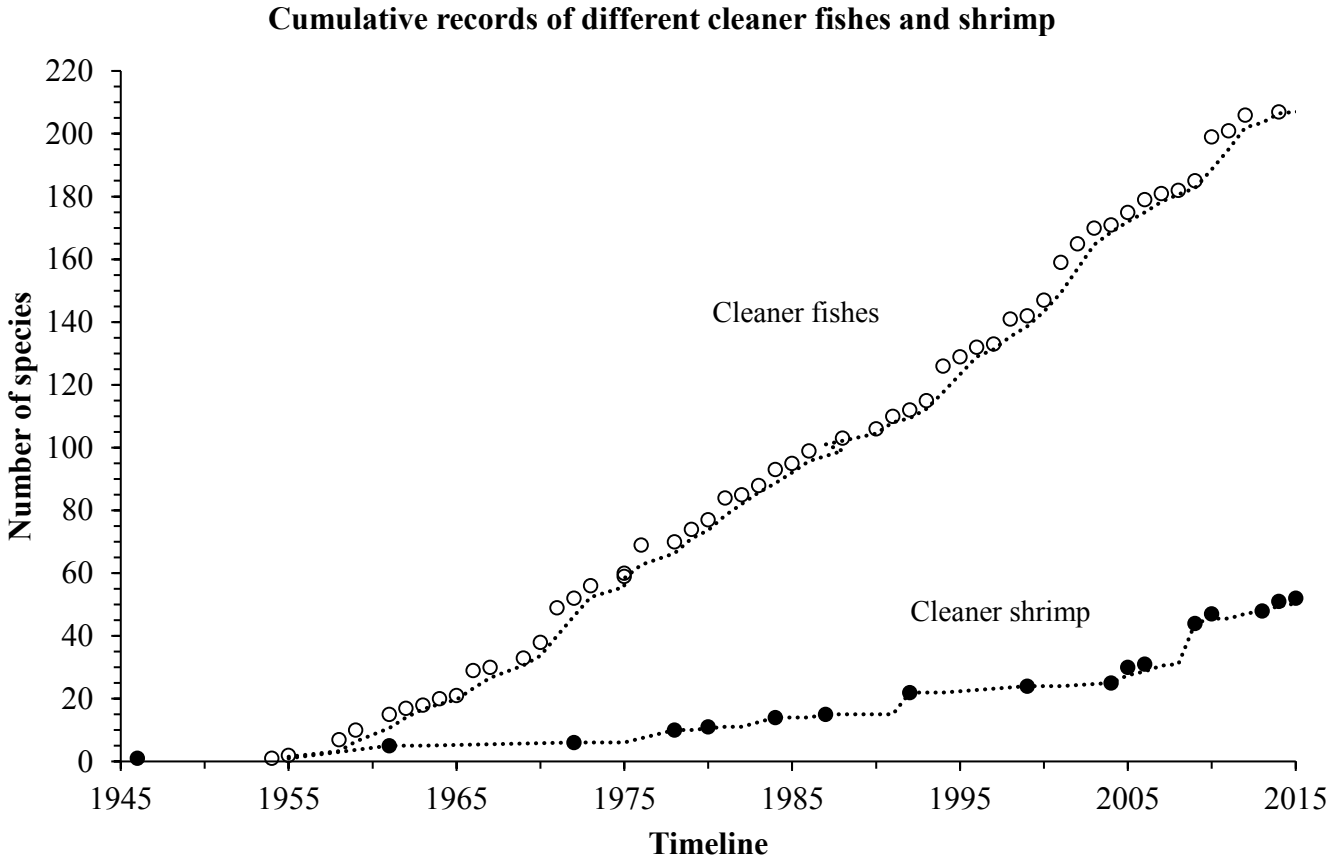

Fig. 2.2. Cumulative records of different cleaner fishes and shrimp.

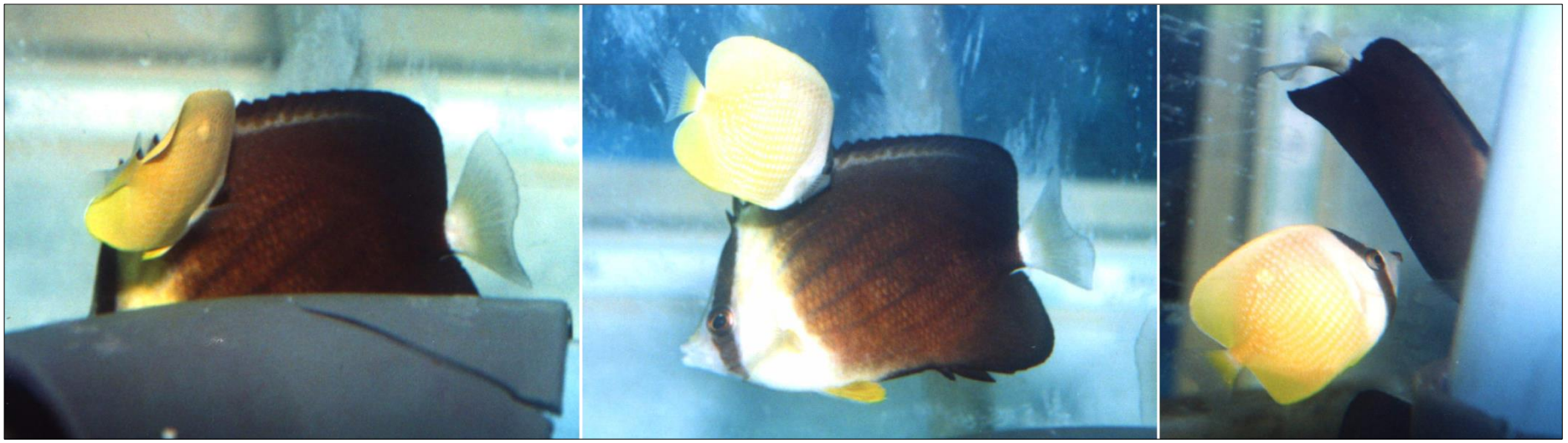

Fig. 2.3. Juvenile *Chaetodon kleinii* Bloch, 1790 cleaning *Chaetodon blackburnii* Desjardins, 1836 infested with *Amyloodinium ocellatum* (E. Brown) E. Brown & Hovasse, 1946.

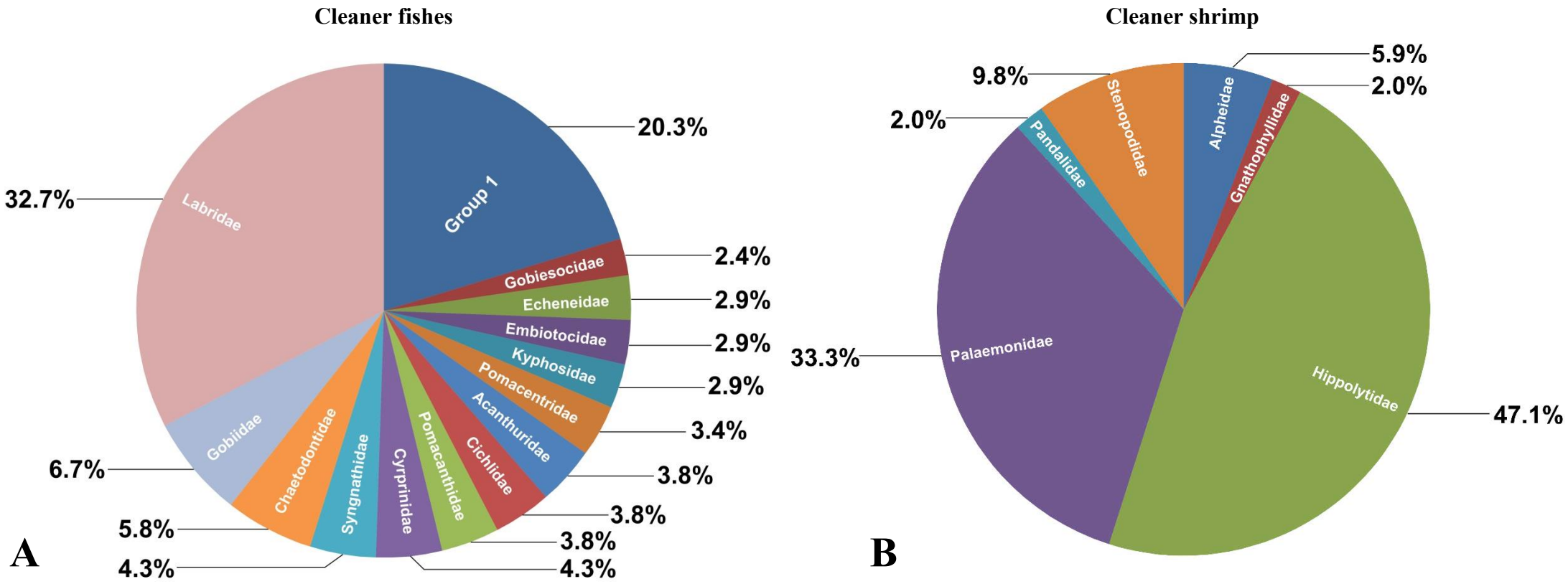

Fig. 2.4. Representation of all known cleaners, at family-level (see Tables 2.1, 2.2), expressed as a percentage. Note. This is not a depiction of the percentage of species in a family that are cleaners. **A**. Group 1 (1–4 species per family): Apogonidae, Balistidae, Cyprinodontidae, Doradidae, Enoplosidae, Gasterosteidae, Monodactylidae, Odacidae, Ostraciidae, Percidae, Poeciliidae, Serrasalmidae and Terapontidae – 1 species each; Bleniidae, Callichthyidae, Grammatidae and Haemulidae – 2 species each; Carangidae, Centrarchidae and Tetraodontidae – 3 species each; Fundulidae, Sparidae and Tripterygiidae – 4 species each; Gobiescocidae – 5 species; Echeneidae, Embiotocidae and Kyphosidae – 6 species each; Pomacentridae – 7 species; Acanthuridae, Cichlidae and Pomacanthidae – 8 species each; Cyprinidae and Syngnathidae – 9 species each; Chaetodontidae – 12 species; Gobiidae – 14 species; Labridae – 68 species. **B**. Alpheidae – 3 species; Gnathophyllidae and Pandalidae – 1 species each; Hippolytidae – 24 species; Palaemonidae – 17 species; Stenopodidae – 5 species.

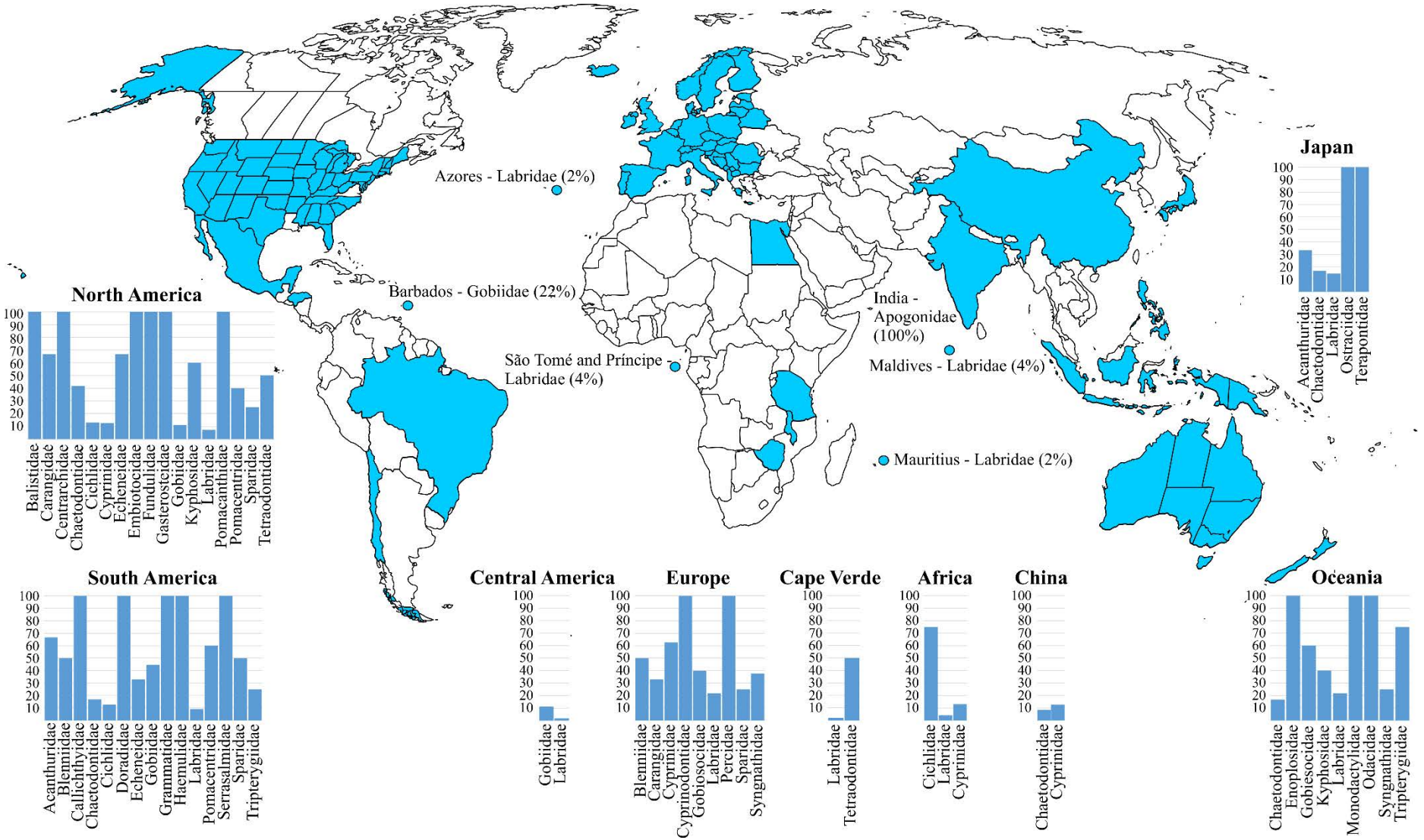

Fig. 2.5. Cleaner fishes reported per region from the research cited in Table 2.1, expressed as a percentage of the total per family. Note: this is not a depiction of regional diversity or taxa distributions, rather an estimate of regional research to demonstrate understudied areas for future focus.

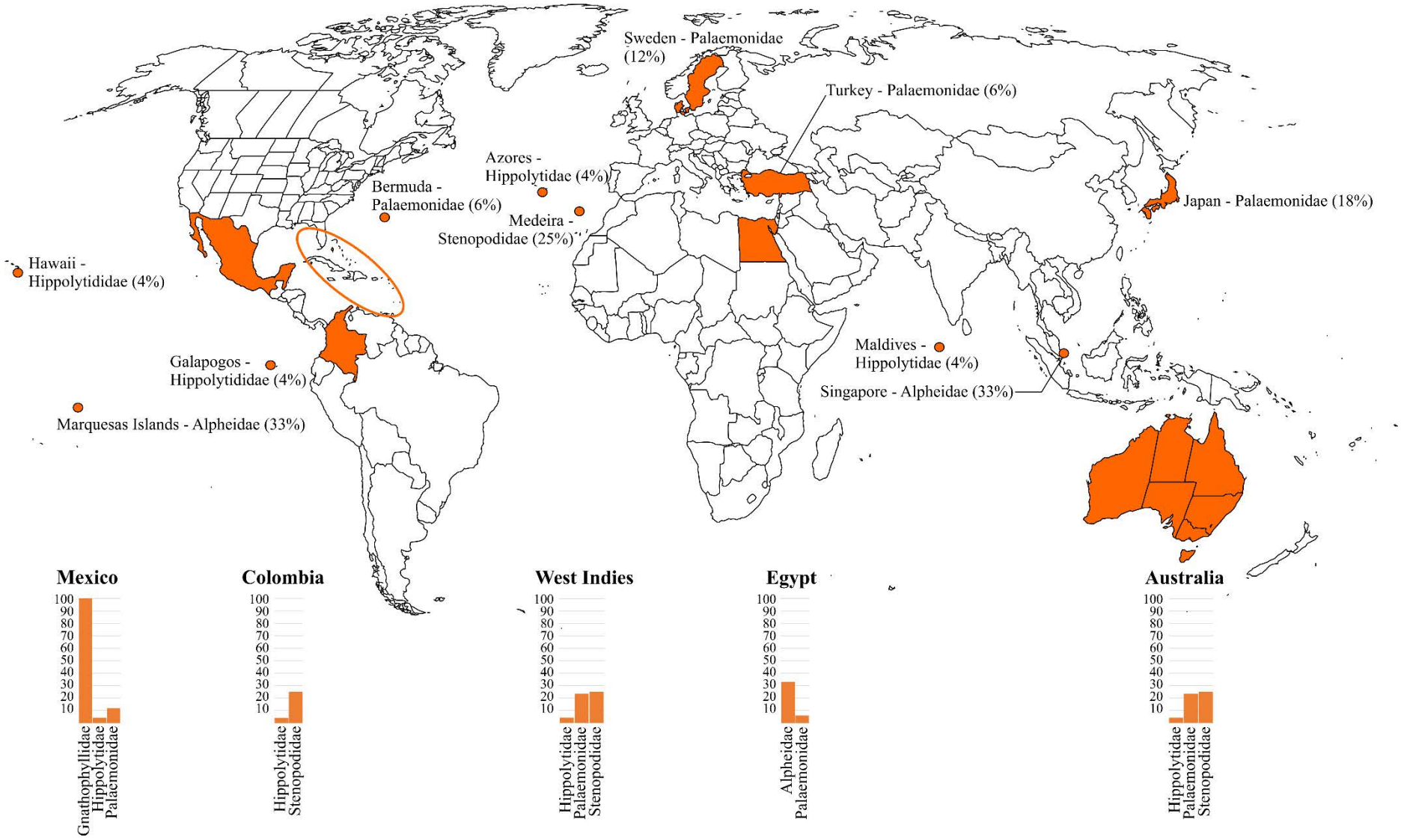

Fig. 2.6. Cleaner shrimp reported per region from the research cited in Table 2.2, expressed as a percentage of the total per family. Note: this is not a depiction of regional diversity or taxa distributions, rather an estimate of regional research to demonstrate understudied areas for future focus.

Table 2.1. List of fishes currently considered as cleaners*.

Family (listed alphabetically)	Representative taxa	Ontogenetic stage	Cleaning lifestyle	Distribution**	References
Acanthuridae	*Acanthurus chirurgus* (Bloch, 1787)	Unreported	Facultative	Western Atlantic: Massachusetts (USA); Bermuda; northern Gulf of Mexico to São Paulo, Brazil. Eastern Atlantic: Senegal	Sazima *et al*. (2010)
	Acanthurus coeruleus Bloch & Schneider, 1801	Unreported	Facultative	Western Atlantic: New York (USA); Bermuda to the Gulf of Mexico and Brazil. Eastern Atlantic: Ascension Island[1]	Sazima *et al*. (2010)
	Acanthurus nigricans (Linnaeus, 1758)	Unreported	Facultative	Eastern Indian Ocean: Cocos-Keeling and Christmas Islands. Pacific Ocean: Ryukyu Islands and Great Barrier Reef to the Hawaiian Islands and French Polynesia	Sazima *et al*. (2010)
	Acanthurus nigrofuscus (Forsskål, 1775)	Unreported	Facultative	Indo-Pacific: Red Sea south to Transkei (South Africa)[2] and east to Hawaiian and Tuamoto islands, north to southern Japan, south to the southern Great Barrier Reef, New Caledonia, and Rapa Island	Sazima *et al*. (2010)
	Ctenochaetus strigosus (Bennett, 1828)	Unreported	Facultative	Eastern Central Pacific: Hawaiian and Johnston Islands. Western Central Pacific: Australia[3]	Sazima *et al*. (2010)
	Zebrasoma flavescens (Bennett, 1828)	Unreported	Facultative	Pacific Ocean: Ryukyu, Mariana, Marshall, Marcus, Wake, Hawaiian Islands.	Sazima *et al*. (2010)
	Prionurus microlepidotus Lacepède, 1804	Juvenile	Facultative	Western Pacific: eastern Australia, including Lord Howe Island[3]and Japan[4]	Kuwamura (1976)
	Prionurus scalprum Valenciennes, 1835***	Unreported	Facultative	Northwest Pacific: Matsushima Bay (Japan) to Taiwan	Shigeta *et al*. (2001)

Table 2.1 cont.

Apogonidae	*Siphamia tubifer* Weber, 1909	Unreported	Facultative	Indo-West Pacific: Red Sea south to Madagascar, east through the Maldives; Sri Lanka and India to the Andaman Sea Islands and Western Australia; north to Ryukyus; throughout Indo-Malayan region to Vanuatu	Eibl-Eibesfldt (1961)
Balistidae	*Canthidermis maculata* (Bloch, 1786)	Unreported	Facultative	Circumglobal excluding Mediterranean Sea	Gooding (1964)
Blenniidae	*Ophioblennius trinitatis* Miranda Ribeiro, 1919	Unreported	Facultative	Southwest Atlantic	Sazima *et al*. (2010)
	Parablennius rouxi (Cocco, 1833)	Unreported	Facultative	Northeast Atlantic: Portugal; northern Mediterranean	Moosleitner (1980)
Callichthyidae	*Corydoras aeneus* (Gill, 1858)	Unreported	Facultative	South America: Colombia; Trinidad to La Plata River basin	Feder (1966)
	Corydoras paleatus (Jenyns, 1842)	Unreported	Facultative	South America: Lower Paraná River basin and coastal rivers, Uruguay and Brazil	Wickler (1956)
Carangidae	*Caranx ruber* (Bloch, 1793)	Juvenile	Facultative	Western Atlantic: New Jersey (USA), Bermuda, and Gulf of Mexico to southern Brazil; Caribbean Sea[5]	Randall (1962)
	Naucrates ductor (Linnaeus, 1758)	Adult	Facultative	Circumtropical	Von Walhert and von Wahlert (1961)
	Oligoplites saurus (Bloch & Schneider, 1801)	Juvenile	Facultative	Western Atlantic: Maine (USA) and northern Gulf of Mexico to Uruguay; West Indies [6]	Carr and Adams (1972); Lucas and Benkert (1983)
Centrarchidae	*Lepomis macrochirus* Rafinesque, 1819	Unreported	Facultative	North America	Sulak (1975)
	Lepomis megalotis (Rafinesque, 1820)	Unreported	Facultative	North America	Spall (1970)

Table 2.1 cont.

	Pomoxis annularis Rafinesque, 1818	Unreported	Facultative	North America	Spall (1970)
Chaetodontidae	*Chaetodon citrinellus* Cuvier, 1831	Juvenile	Facultative	Indo-Pacific: East Africa to the Hawaiian, Marquesan and Tuamoto Islands, north Japan and Ogasawara Islands, south to New South Wales (Australia) and Lord Howe Island	Grutter (2002)
	Chaetodon kleinii Bloch, 1790	Juvenile	Facultative	Indo-Pacific: Red Sea and East Africa to the Hawaiian Islands and Samoa, north to southern Japan, south to New South Wales (Australia); New Caledonia. Eastern Pacific: Galapagos Islands	Personal observations (**Fig. 2.3**)
	Chaetodon litus Randall & Caldwell, 1973	Juvenile	Facultative	Southeast Pacific: Easter Island	Allen (1978)
	Chaetodon miliaris Quoy & Gaimard, 1825	Unreported	Facultative	Eastern Central Pacific: Johnston Island[7]; Hawaii	Bennett and Keuper-Bennett (1995)
	Chaetodon plebeius Cuvier, 1831	Unreported	Facultative	Western Pacific: Andaman Sea to Fiji, north to Japan, south to Australia; Tonga[8]	Sadovy and Cornish (2000)
	Chaetodon striatus Linnaeus, 1758	Adult	Facultative	Western Atlantic: Massachusetts (USA) to Santa Catarina, Brazil[9]; Gulf of Mexico; Caribbean Sea. Eastern Central Atlantic: St. Paul's Rocks[10]	Maia-Nogueira *et al*. (2010)
	Forcipiger flavissimus Jordan & McGregor, 1898	Adult	Facultative	Indo-Pacific: Red Sea; East Africa[11] to Hawaiian and Easter Islands, north to southern Japan, south to Lord Howe Island; Micronesia. Eastern Pacific: southern Baja California (Mexico); Revillagigedo and Galapagos Islands	Craig (2007)

Table 2.1 cont.

	Heniochus acuminatus (Linnaeus, 1758)	Unreported	Facultative	Indo-Pacific: East Africa and Persian Gulf to Society Islands, north to southern Japan, south to Lord Howe Island; Micronesia	Shigeta *et al*. (2001)
	Heniochus diphreutes Jordan, 1903	Juvenile	Facultative	Indo-Pacific: Red Sea; South Africa to warm-temperate Australia; Hawaii	Randall (1985)
	Heniochus monoceros Cuvier, 1831	Unreported	Facultative	Indo-Pacific: East Africa to Tuamoto Islands, north to southern Japan, south to New South Wales (Australia); Tonga	Shigeta *et al*. (2001)
	Johnrandallia nigrirostris (Gill, 1862)	Adult and juvenile	Facultative	Eastern Pacific: Gulf of California to Panama; Cocos, Malpelo and Galapagos Islands	Feder (1966)
	Prognathodes falcifer (Hubbs & Rechnitzer, 1958)	Unreported (~150mm)	Facultative	Eastern Pacific: Santa Catalina Island, southern California (USA) to Peru; Galapagos Islands	Lea and Richards (2005)
Cichlidae	*Docimodus evelynae* Eccles & Lewis, 1976	Sub adult	Facultative	Africa: Lake Malawi	Ribbink (1983)
	Etroplus maculatus (Bloch, 1795)	Juvenile	Facultative	Asia: India and Sri Lanka	Wyman and Ward (1972)
	Maylandia pursa (Stauffer, 1991)	Unreported	Facultative	Africa: Lake Malawi	Stauffer (1991)
	Mesonauta festivus (Heckel, 1840)	Unreported	Facultative	South America: Bolivia, Brazil, Paraguay, Peru	Severo-Neto and Froehlich (2016)
	Pseudotropheus crabro (Ribbink & Lewis, 1982)	Unreported	Facultative	Africa: Lake Malawi	Ribbink and Lewis (1982)
	Haplochromis cnester Witte & Witte-Maas, 1981	Unreported	Facultative	Africa: Lake Victoria	Witte and Witte-Maas (1981)
	Haplochromis teunisrasi Witte & Witte-Maas, 1981	Unreported	Facultative	Africa: Lake Victoria	Witte and Witte-Maas (1981)

Table 2.1 cont.

	Melanochromis loriae Johnson, 1975	Unreported	Facultative	Africa: Lake Malawi	Van Tassell *et al.* (1994)
Cyprinidae	*Alburnus alburnus* (Linnaeus, 1758)	Unreported	Facultative	Europe and Asia	Abel (1971)
	Barbus tetrazona (Bleeker, 1855)	Unreported	Facultative	Asia: Sumatra and Borneo	Darkhov and Panyushkin (1988)
	Cyprinus carpio Linnaeus, 1758	Unreported	Facultative	Europe and Asia	Soto *et al.* (1994)
	Labeo cylindricus Peters, 1852	Juvenile	Facultative	Africa	Minshull (1985)
	Pimephales promelas Rafinesque, 1820	Unreported	Facultative	North America	Spall (1970)
	Rhodeus amarus (Bloch, 1782)	Unreported	Facultative	Europe: central and eastern Europe and northern Asia Minor[12]	Abel (1971)
	Rutilus rutilus (Linnaeus, 1758)	Unreported	Facultative	Europe	Abel (1971)
	Scardinius erythrophthalmus (Linnaeus, 1758)	Unreported	Facultative	Eurasia: European rivers north of Pyrenees and Alps, eastward to Ural and Eya drainages, Aral and White Sea basins; Black Sea basin in Europe; northern Asia Minor	Abel (1971)
	Tinca tinca (Linnaeus, 1758)	Unreported	Facultative	Eurasia	Abel (1971)
Cyprinodontidae	*Cyprinodon variegatus* Lacepède, 1803	Adult and juvenile	Facultative	North and South America: Massachusetts (USA) to northeastern Mexico; West Indies[13]; Bahamas, Antilles, Gulf of Mexico, Yucatan, Venezuela[14]	Able (1976)
Doradidae	*Platydoras costatus* (Linnaeus, 1758)	Juvenile	Facultative	South America: Amazon, Tocantins, Parnaíba, Orinoco, Essequibo River basins and coastal drainages in French Guiana and Suriname[15]; Argentina[16]	Carvalho *et al.* (2003)
Echeneidae	*Echeneis naucrates* Linnaeus, 1758	Adult and juvenile	Facultative	Circumtropical	Arnal *et al.* (2006)

Table 2.1 cont.

	Phtheirichthys lineatus (Menzies, 1791)	Unreported	Facultative	Circumtropical/subtropical	Cressey and Lechner (1970)
	Remora australis (Bennett, 1840)	Unreported	Facultative	Circumtropical	Sazima *et al*. (2006)
	Remora brachyptera (Lowe, 1839)	Unreported	Facultative	Circumtropical	Strasburg (1959)
	Remora osteochir (Cuvier, 1829)	Unreported	Facultative	Circumtropical/temperate	Cressey and Lechner (1970)
	Remora remora (Linnaeus, 1758)	Adult and juvenile	Facultative	Circumtropical/temperate	Szidet and Nani (1951); Strassburg (1959)
Embiotocidae	*Brachyistius frenatus* Gill, 1862	Unreported	Facultative	Eastern Pacific: northern British Columbia (Canada) to central Baja California (Mexico); Guadalupe Island	Hubbs and Hubbs (1954)
	Embiotoca jacksoni Agassiz, 1853	Unreported	Facultative	Eastern Pacific: northern California (USA) to central Baja California (Mexico); Guadalupe Island	Hobson (1969)
	Hypsurus caryi (Agassiz, 1853)	Unreported	Facultative	Eastern Pacific: northern California (USA) to northern Baja California (Mexico)	Gotshall (1967)
	Phanerodon atripes (Jordan & Gilbert, 1880)	Unreported	Facultative	Eastern Pacific: northern California (USA) to central Baja California (Mexico)	Hobson (1969)
	Phanerodon furcatus Girard, 1854	Unreported	Facultative	Eastern Pacific: Vancouver Island, southern British Columbia (Canada) to Punta Cabras, northern Baja California (Mexico)	Hobson (1971)
	Rhacochilus vacca (Girard, 1855)	Juvenile	Facultative	Eastern Pacific: southeastern Alaska to Guadalupe Island (Mexico)	Gotshall (1967); Van Tassell *et al.* (1994)
Enoplosidae	*Enoplosus armatus* (White, 1790)	Unreported	Facultative	Australia	Shepherd *et al.* (2005)
Fundulidae	*Adinia xenica* (Jordan & Gilbert, 1882)	Unreported	Facultative	Western Central Atlantic: USA	Able (1976)

Table 2.1 cont.

	Fundulus heteroclitus (Linnaeus, 1766)	Unreported	Facultative	Western Atlantic: Gulf of St. Lawrence to northeast Florida (USA)[17]	McCutcheon and McCutcheon (1964); Feder (1966)
	Fundulus majalis (Walbaum, 1792)	Adult and juvenile	Facultative	Western Atlantic: New Hampshire to northeastern Florida (USA); northern Gulf of Mexico	Able (1976)
	Lucania parva (Baird & Girard, 1855)	Adult and juvenile	Facultative	Western Atlantic: Massachusetts and northern Gulf of Mexico to Florida Key (USA) and northeastern Mexico[18]	Able (1976)
Gasterosteidae	*Apeltes quadracus* (Mitchill, 1815)	Unreported	Facultative	Western Atlantic: Gulf of St. Lawrence (Canada) to North Carolina (USA)[17]	Tyler (1963); Able (1976)
Gobiesocidae	*Cochleoceps bicolor* Hutchins, 1991	Unreported	Facultative	Eastern Indian Ocean: southern Australia	Hutchins (1991)
	Cochleoceps orientalis Hutchins, 1991	Unreported	Facultative	Southwest Pacific: New South Wales, eastern Victoria (Australia)	Hutchins (1991)
	Cochleoceps viridis Hutchins, 1991	Unreported	Facultative	Eastern Indian Ocean: southwestern coast of Australia	Hutchins (1991)
	Diplecogaster bimaculata (Bonnaterre, 1788)	Unreported	Facultative	Aegean Sea, Black Sea, Sea of Marmara, Mediterranean Sea; NE Atlantic	Patzner and Debelius, (1984)
	Lepadogaster candolii Risso, 1810	Unreported	Facultative	Eastern Atlantic: British Isles to the Canary Islands including western Mediterranean and the Black Sea	Weitzman and Mercader (2012)
Gobiidae	*Elacatinus evelynae* (Böhlke & Robins, 1968)	Unreported	Dedicated	Western Atlantic: Bahamas, Antilles to the northern coast of South America; Western Caribbean[14]	Whiteman and Côté (2002)
	Elacatinus figaro Sazima, Moura & Rosa, 1997	Unreported	Dedicated	Southwest Atlantic: Brazil	Sazima *et al.* (2000)
	Elacatinus genie (Böhlke & Robins, 1968)	Unreported	Dedicated	Western Central Atlantic: Bahamas; Grand Cayman Island	Colin (1975)

Table 2.1 cont.

	Elacatinus illecebrosum (Böhlke & Robins, 1968)	Unreported	Dedicated	Western Central Atlantic: Yucatan, Mexico to Panama	Colin (1975)
	Elacatinus lobeli Randall & Colin, 2009	Adult	Dedicated	Western Central Atlantic: Caribbean Sea. Belize, Honduras	Randall and Colin (2009)
	Elacatinus oceanops Jordan, 1904	Unreported	Dedicated	Western Central Atlantic: southern Florida to Texas (USA) southward to Belize	Randall (1958)
	Elacatinus phthirophagus Sazima, Carvalho-Filho & Sazima, 2008	Unreported	Dedicated	Atlantic Ocean: Fernando de Noronha Archipelago[19]	Sazima *et al.* (2008)
	Elacatinus pridisi Guimarães, Gasparini & Rocha, 2004	Unreported	Dedicated	Southwest Atlantic: Trinidade Island (Brazil)	Guimarães *et al.* (2004)
	Elacatinus prochilos (Böhlke & Robins, 1968)	Unreported	Dedicated	Western Central Atlantic: southern Florida (USA); Lesser Antilles	Whiteman and Côté (2002)
	Elacatinus puncticulatus (Ginsburg, 1938)	Unreported	Facultative	Eastern Central Pacific: Gulf of California to Ecuador	Feder (1966)
	Elacatinus randalli (Böhlke & Robins, 1968)	Unreported	Dedicated	Western Central Atlantic: Puerto Rico and the Lesser Antilles to Curaçao and Venezuela	Sazima and Moura (2000)
	Tigrigobius digueti (Pellegrin, 1901)	Unreported	Facultative	Eastern Central Pacific: Gulf of California to Colombia	Hobson (1969)
	Tigrigobius limbaughi (Hoese & Reader, 2001)	Unreported	Facultative	Eastern Central Pacific: Mexico	Hoese and Reader (2001)
	Tigrigobius nesiotes (Bussing, 1990)	Unreported	Facultative	Eastern Central Pacific. Costa Rica	Grove and Lavenberg (1997)
Grammatidae	*Gramma loreto* Poey, 1868	Unreported	Facultative	Western Central Atlantic: Bermuda, Bahamas, and Central America to northern South America	Böhlke and Chaplin (1993)

Table 2.1 cont.

	Gramma brasiliensis Sazima, Gasparini & Moura, 1998	Unreported	Facultative	Southwest Atlantic: Brazil	Sazima *et al.* (1998a)
Haemulidae	*Anisotremus virginicus* (Linnaeus, 1758)	Unreported (~150mm)	Facultative	Western Atlantic: Bermuda, Florida (USA) to Brazil, including the Gulf of Mexico and the Caribbean Sea[20]	Sazima *et al*. (2010)
	Haemulon californiensis (Steindachner, 1876)	Juvenile	Facultative	Eastern Pacific: California (USA) to Peru	Sikkel (1986)
	Haemulon chrysargyreum Günther, 1859	Unreported	Facultative	Western Atlantic: southern Florida (USA), Bahamas and Yucatan, Mexico to Brazil	Sazima *et al*. (2010)
Kyphosidae	*Atypichthys strigatus* (Günther, 1860)	Unreported	Facultative	Indo-West Pacific: southeastern Australia	Glasby and Kingsford (1994)
	Girella nigricans (Ayres, 1860)	Unreported	Facultative	Eastern Central Pacific: San Francisco in California (USA) to southern Baja California (Mexico)	DeMartini and Coyer (1981); McCourt and Thomson (1984)
	Girella simplicidens Osburn & Nichols, 1916	Unreported	Facultative	Eastern Central Pacific: Gulf of California	McCourt and Thomson (1984)
	Hermosilla azurea Jenkins & Evermann, 1889	Unreported	Facultative	Eastern Central Pacific: Monterey Bay in California (USA) to Gulf of California	DeMartini and Coyer (1981); McCourt and Thomson (1984)
	Medialuna californiensis (Steindachner, 1876)	Unreported	Facultative	Eastern Pacific: Vancouver Island (Canada) to Gulf of California (USA)	Hixon (1979)
	Tilodon sexfasciatus (Richardson, 1842)	Unreported	Facultative	Southern Australia	Shepherd *et al.* (2005)
Labridae	*Austrolabrus maculatus* (Macleay, 1881)	Unreported	Facultative	Australia: Western Australia to New South Wales	Shepherd *et al.* (2005)

Table 2.1 cont.

Bodianus anthioides (Bennett, 1832)	Juvenile	Facultative	Indo-Pacific: Red Sea to South Africa[21]; east to Line and Tuamoto Islands, north to southern Japan, south to New Caledonia and the Austral Islands	Bshary (2003)
Bodianus axillaris (Bennett, 1832)	Juvenile	Facultative	Indo-Pacific: Red Sea to South Africa[21]; Marshall, Marquesan and Tuamoto Islands, and north to Japan[22]	Randall (1992)
Bodianus diana (Lacepède, 1801)	Juvenile	Facultative	Indian Ocean: East Africa, east to the Nicobar Islands and Cocos-Keeling Islands[23]	Randall (1992)
Bodianus diplotaenia (Gill, 1862)	Juvenile	Facultative	Eastern Pacific: Guadalupe Island, Gulf of California to Chile, Cocos, Malpelo, Revillagigedo and Galapagos Islands	Feder (1966)
Bodianus mesothorax (Bloch & Schneider, 1801)	Juvenile	Facultative	Western Pacific between Wakayama Prefecture (Japan) and Sydney (Australia), New Caledonia and Fiji. Indian Ocean; western coast of Malaysia and Indonesia, and Nicobar Islands	Wicksten (1998); Côté (2000)
Bodianus pulchellus (Poey, 1860)	Juvenile	Facultative	Western Atlantic: South Carolina, USA and Bermuda to Honduras and Santa Catarina (Brazil)[9]. Eastern Atlantic: São Tomé Island[24]	Randall (1962); Afonso *et al.* (1999); Quimbayo *et al*. (2012)
Bodianus rufus (Linnaeus, 1758)	Juvenile	Facultative	Western Atlantic: Bermuda, southern Florida (USA), Gulf of Mexico and Caribbean Sea to southern Brazil	Eibl-Eibesfeldt (1955); Limbaugh (1961)
Bodianus speciosus (Bowdich, 1825)	Juvenile	Facultative	Eastern Central Atlantic: tropical western coast of Africa, from Cameroon to Guinea Cape Verde Islands	Afonso *et al.* (1999); Quimbayo *et al*. (2012)
Centrolabrus caeruleus Azevedo, 1999	Unreported	Facultative	Northeast Atlantic: Azores Islands	Bertoncini *et al.* (2009)
Centrolabrus exoletus (Linnaeus, 1758)	Adult and juvenile	Facultative	Eastern Atlantic: Norway to Portugal; eastern Greenland	Galeote and Otero (1998)

Table 2.1 cont.

Coris atlantica Günther, 1862	Unreported	Facultative	Eastern Atlantic: Cape Verde to Liberia	Quimbayo *et al*. (2012)
Coris musume (Jordan & Snyder, 1904)	Unreported	Facultative	Northwest Pacific: southern Japan, Izu Islands, and Taiwan	Hirata *et al.* (1996); Shigeta *et al*. (2001)
Coris julis (Linnaeus, 1758)	Juvenile	Facultative	Eastern Atlantic: Sweden to south of Cape Lopez, Gabon, Mediterranean Sea	Arnal *et al.* (2006)
Coris picta (Bloch & Schneider, 1801)	Adult and juvenile	Facultative	Western Pacific: southern Queensland to northern Victoria (Australia); Lord Howe Island, Norfolk and Kermadec Islands, New Zealand	Ayling and Grace (1971); Côté (2000)
Coris sandageri (Hector, 1884)	Juvenile and sub-adult	Facultative	Southwest Pacific: Australia and New Zealand, including Lord Howe, Norfolk and Kermadec Islands	Ayling and Grace (1971); Côté (2000)
Ctenolabrus rupestris (Linnaeus, 1758)	Adults?	Facultative	Eastern Atlantic: Norway to Morocco. Also from Mediterranean and Black Seas	Hilldén (1983); Arnal *et al.* (2006)
Diproctacanthus xanthurus (Bleeker, 1856)	Adult and juvenile	Dedicated	Western Central Pacific: Philippines, Palau, Indonesia, New Guinea, Great Barrier Reef	Randall *et al*. (1990)
Halichoeres bivittatus (Bloch, 1791)	Juvenile	Facultative	Western Atlantic: North Carolina (USA), Bermuda to Brazil[25]	Côté (2000)
Halichoeres bleekeri (Steindachner & Döderlein, 1887)	Unreported	Facultative	Western Pacific: Japan to the Philippines	Shigeta *et al.* (2001)
Halichoeres brasiliensis (Bloch, 1791)	Unreported	Facultative	Southwest Atlantic: Brazil and Trinidad	Sazima *et al*. (1998b); Côté (2000)
Halichoeres cyanocephalus (Bloch, 1791)	Juvenile	Facultative	Western Atlantic: Florida (USA), Antilles to Brazil	Sazima *et al.* (1998b); Côté (2000)
Halichoeres nicholsi (Jordan & Gilbert, 1882)	Juvenile	Facultative	Indo-West Pacific: Red Sea, Gulf to Samoa, north to southern Japan, south to the Great Barrier Reef.	McCourt and Thomson (1984)

Table 2.1 cont.

Halichoeres poeyi (Steindachner, 1867)	Unreported	Facultative	Western Atlantic: southern Florida (USA), Bahamas to São Paulo, Brazil[9]	Sazima *et al.* (1998b)
Halichoeres semicinctus (Ayres, 1859)	Unreported	Facultative	Eastern Pacific: Point Conception (California, USA), Guadalupe Island (Baja California), Gulf of California (Mexico)	Hobson (1976); Sazima *et al.* (1998b); Gomon (1995)
Halichoeres zeylonicus (Bennett, 1833)	Juvenile	Facultative	Indo-West Pacific: Red Sea, Gulf to Samoa, north to southern Japan, south to the Great Barrier Reef[26]	Clark and Petzold (1998)
Larabicus quadrilineatus (Rüppell, 1835)	Juvenile	Facultative	Western Indian Ocean: Red Sea; Gulf of Aden	Randall (1986a)
Labrichthys unilineatus (Guichenot, 1847)	Juvenile	Facultative	Indo-Pacific: East Africa to Micronesia and Samoa	Debelius (1993); Shigeta *et al*. (2001)
Labroides bicolor Fowler & Bean, 1928	Adult and juvenile	Dedicated	Indo-Pacific: East Africa to Line, Marquesan and Society Islands, north to Japan, south to Lord Howe Island	Randall (1958)
Labroides dimidiatus (Valenciennes, 1839)	Adult and juvenile	Dedicated	Indo-Pacific: Red Sea and East Africa[27], to Line, Marquesas, and Ducie Islands, north to southern Japan, south to Lord Howe and Rapa Islands	Randall (1958), Randall *et al.* (1990)
Labroides phthirophagus Randall, 1958	Adult and juvenile	Dedicated	Eastern Central Pacific: Hawaiian Islands[28] and the Johnston Islands[29]	Randall (1958)
Labroides pectoralis Randall & Springer, 1975	Adult and juvenile	Dedicated	Pacific Ocean: Cocos-Keeling Island to Line and Pitcairn Islands, north to Bonin Islands, south to Rowley Shoals and Great Barrier Reef	Randall and Springer (1975); Shigeta *et al*. (2001)
Labroides rubrolabiatus Randall, 1958	Adult and juvenile	Dedicated	Eastern Central Pacific: Samoa to Line and Society Islands, French Polynesia and Pitcairn	Randall (1958)
Labropsis alleni Randall, 1981	Juvenile	Facultative	Western Central Pacific: Indonesia, Philippines, New Guinea, Solomon Islands, Palau, Marshall Islands	Randall (1981); Cole (2010)

Table 2.1 cont.

Labropsis australis Randall, 1981	Juvenile	Facultative	Western Pacific: Solomon Islands, Samoa Islands, Vanuatu, Fiji, Loyalty Islands (New Caledonia), Tonga, Great Barrier Reef	Randall (1981); Westneat (2001)
Labropsis manabei Schmidt, 1931	Adult	Facultative	Eastern Indian Ocean: Scott Reef[30] and Western Pacific: Japan, Taiwan, Philippines	Masuda and Kobayashi (1994); Kuiter and Tonozuka (2001a); Shigeta *et al.* (2001)
Labropsis micronesica Randall, 1981	Juvenile	Facultative	Western Central Pacific: Belau, Caroline, Mariana and Marshall Islands	Randall (1981)
Labropsis xanthonota Randall, 1981	Juvenile	Facultative	Indo-Pacific: East Africa to Samoa, north to Izu Islands, south to Great Barrier Reef	Myers (1991)
Labrus bergylta Ascanius, 1767	Juvenile	Facultative	Eastern Atlantic: Norway to Morocco; Madeira, Azores and Canary Islands	Skiftesvik *et al*. (2014)
Labrus mixtus Linnaeus, 1758	Adult (female)	Facultative	Eastern Atlantic: Norway to Senegal; Azores; Madeira; Mediterranean Sea	Bjordal (1988)
Oxyjulis californica (Günther, 1861)	Adult and juvenile	Facultative	Eastern Pacific: Salt Point, California (USA) to southern central Baja California (Mexico)	Limbaugh (1961)
Pseudocheilinus hexataenia (Bleeker, 1857)	Juvenile	Facultative	Indo-Pacific: Red Sea south to Natal (South Africa)[27], east to the Tuamoto Islands, north to the Ryukyu Islands, south to Lord Howe and the Austral Islands	Barbu *et al.* (2011)
Pseudocheilinus octotaenia Jenkins, 1901	Unreported	Facultative	Indo-Pacific: East Africa to Hawaiian and Ducie Islands, north to Yaeyama Island	Bennett and Keuper-Bennett (1995)
Pseudodax moluccanus (Valenciennes, 1840)	Unreported	Facultative	Indo-Pacific: Red Sea to South Africa[21], to Society, Marquesan, Tuamoto Islands and north to Japan	Randall *et al*. (1990)

Table 2.1 cont.

Pseudolabrus japonicus (Houttuyn, 1782)	Unreported	Facultative	Northwest Pacific: central Honshu to Okinawajima, Japan; South Korea; southern China; Taiwan; Hong Kong	Shigeta *et al*. (2001)
Pseudolabrus luculentus (Richardson, 1848)	Juvenile, sub-adult (female)?	Facultative	Southwest Pacific: Australia; Lord Howe and Norfolk Islands; New Zealand; Kermadec Islands	Ayling and Grace (1971)
Pseudolabrus miles (Schneider & Forster, 1801)	Juvenile	Facultative	Southwest Pacific: New Zealand; Snares, Stewart, Clatham and Three Kings Islands	Ayling and Grace (1971)
Scarus zelindae Moura, Figueiredo & Sazima, 2001	Unreported	Facultative	Southwest Atlantic: Brazil	Sazima *et al*. (2010)
Semicossyphus pulcher (Ayres, 1854)	Unreported	Facultative	Eastern Pacific: Monterey Bay, California (USA) to Guadalupe Island; Gulf of California	McCourt and Thomson (1984)
Suezichthys aylingi Russell, 1985	Adult and juvenile	Facultative	Southwest Pacific: southeastern Australia and northeastern New Zealand	Hutchins and Swainston (1986)
Symphodus mediterraneus (Linnaeus, 1758)	Juvenile	Facultative	Eastern Atlantic: Portugal to northern Morocco, Azores, Madeira and Mediterranean Sea	Zander and Sötje (2002)
Symphodus melanocercus (Risso, 1810)	Adult?	Facultative	Mediterranean Sea	Von Wahlert and von Wahlert (1961); Flückiger (1981)
Symphodus melops (Linnaeus, 1758)	Unreported	Facultative	Eastern Atlantic: Norway to Morocco and the Azores; Mediterranean and Adriatic seas	Bjordal (1988)
Symphodus ocellatus (Linnaeus, 1758)	Adult (female)	Facultative	Eastern Atlantic: Mediterranean, Black Sea and Sea of Azov	Zander and Sötje (2002)
Symphodus tinca (Linnaeus, 1758)	Juvenile	Facultative	Eastern Atlantic: Spain to Morocco, Mediterranean and Black Seas	Zander and Sötje (2002)

Table 2.1 cont.

Symphodus roissali (Risso, 1810)	Unreported	Facultative	Eastern Atlantic: Gulf of Gascogne to Gibraltar; Mediterranean and Black Seas	Arnal *et al.* 2006
Symphodus rostratus (Bloch, 1791)	Unreported	Facultative	Eastern Atlantic: Mediterranean and western part of Black Sea	Potts (1973)
Thalassoma amblycephalum (Bleeker, 1856)	Unreported	Facultative	Indo-Pacific: Somalia[31]; South Africa[27] to Line, Marquesan, and Tuamoto Islands, north to southern Japan, south to Rowley Shoals, northern New Zealand and Lord Howe, Rapa Islands	Debelius (1993); Shigeta *et al*. (2001)
Thalassoma bifasciatum (Bloch, 1791)	Juvenile	Facultative	Western Atlantic: Bermuda; Florida (USA); southeastern Gulf of Mexico; Caribbean Sea to northern South America	Eibl-Eibesfeldt (1955)
Thalassoma cupido (Temminck & Schlegel, 1845)	Sub-adult	Facultative	Northwest Pacific: Japan to Taiwan	Kuwamura (1976)
Thalassoma duperrey (Quoy & Gaimard, 1824)	Adult and juvenile	Facultative	Eastern Central Pacific: Johnston[7] and Hawaiian Islands	Losey *et al*. (1994)
Thalassoma lucasanum (Gill, 1862)	Juvenile	Facultative	Eastern Pacific: Gulf of California to Peru; Galapagos Islands	Feder (1966)
Thalassoma lunare (Linnaeus, 1758)	Juvenile	Facultative	Indo-Pacific: Red Sea and East Africa[27]; Line Islands, north to Japan, south to Lord Howe Island; northern New Zealand[32]	Randall (1986b)
Thalassoma lutescens (Lay & Bennett, 1839)	Juvenile	Facultative	Indo-Pacific: Sri Lanka to Ducie Island, north to Japan and the Hawaiian Islands, south to southeastern Australia, Lord Howe Island, the Kermadec Islands, and Rapa	McCourt and Thomson (1984)
Thalassoma newtoni (Osório, 1891)	Unreported	Facultative	Eastern Atlantic: Sao Tome	Quimbayo *et al*. (2012)

Table 2.1 cont.

	Thalassoma norohanum (Boulenger, 1890)	Adult (small) and juvenile	Facultative	Western Atlantic: Brazil and its oceanic islands	Francini-Filho *et al.* (2000)
	Thalassoma pavo (Linnaeus, 1758)	Juvenile	Facultative	Eastern Atlantic: Portugal to south of Cape Lopez, Gabon; Azores, Madeira, Canary, São Tomé and Annobon Islands; Mediterranean Sea	Moosleitner (1980)
	Thalassoma rueppellii (Klunzinger, 1871)	Juvenile	Facultative	Western Indian Ocean: Red Sea	Randall (1986b)
Monodactylidae	*Monodactylus argenteus* (Linnaeus, 1758)	Unreported	Facultative	Indo-West Pacific: Red Sea and East Africa[33] to Samoa, north to Yaeyamas, south to New Caledonia, Australia	Van Tassell *et al.* (1994)
Odacidae	*Siphonognathus beddomei* (Johnston, 1885)	Adults	Facultative	Eastern Indian Ocean: southern Australia	Kuiter (1996)
Ostraciidae	*Ostracion immaculatus* Temminck & Schlegel, 1850	Unreported	Facultative	Northwest Pacific: Japan	Shigeta *et al.* (2001)
Percidae	*Perca fluviatilis* Linnaeus, 1758	Juvenile	Facultative	Eurasia	Able (1971)
Poeciliidae	*Poecilia reticulata* Peters, 1859	Unreported	Facultative	South America: Venezuela, Barbados, Trinidad, northern Brazil; Guyanas	Darkhov and Panyushkin (1988)
Pomacanthidae	*Holacanthus bermudensis* Goode, 1876	Juvenile	Facultative	Western Atlantic: Bermuda, Bahamas, southern Florida, (USA) to Gulf of Mexico, including Yucatan (Mexico)[14]	Thresher (1979)
	Holacanthus ciliaris (Linnaeus, 1758)	Juvenile	Facultative	Western Atlantic: Florida (USA) and Gulf of Mexico to Brazil. Eastern Central Atlantic: St. Paul's Rocks[10]	Allen (1978)
	Holacanthus limbaughi Baldwin, 1963	Unreported	Facultative	Eastern Pacific: Clipperton Island	Feder (1966)
	Holacanthus passer Valenciennes, 1846	Unreported	Facultative	Eastern Pacific: Gulf of California to Peru; Galapagos Islands	Feder (1966)

Table 2.1 cont.

	Pomacanthus arcuatus (Linnaeus, 1758)	Juvenile	Facultative	Western Atlantic: New England (USA) to Rio de Janeiro, Brazil; Gulf of Mexico; Caribbean[14]	Brockmann and Hailman (1976)
	Pomacanthus imperator (Bloch, 1787)	Unreported	Facultative	Indo-Pacific: Red Sea and East Africa to the Hawaiian, Line and Tuamoto Islands, north to southern Japan and the Ogasawara Islands, south to the Great Barrier Reef, New Caledonia, and the Austral Islands[34]	Shigeta *et al*. (2001)
	Pomacanthus paru (Bloch, 1787)	Unreported	Facultative	Western Atlantic: Florida (USA), Bahamas to Brazil, including Gulf of Mexico and Caribbean[14]	Brockmann and Hailman (1976); Sazima *et al*. (2010)
	Pomacanthus zonipectus (Gill, 1862)	Unreported	Facultative	Eastern Pacific: Gulf of California; north of Bahía Magdalena, Mexico to Peru	McCourt and Thomson (1984)
Pomacentridae	*Abudefduf saxatilis* (Linnaeus, 1758)	Unreported	Facultative	Atlantic Ocean: Canada[35] to Rhode Island (USA) to Uruguay in the western Atlantic. Caribbean; Cape Verde; western Africa to Angola	Sazima *et al*. (2010)
	Abudefduf sexfasciatus (Lacepède, 1801)	Unreported	Facultative	Indo-Pacific: Red Sea to Pinda (Mozambique)[36]; Tuamoto Islands, north to southern Japan, south to Lord Howe and Rapa Islands.	Sazima *et al*. (2010)
	Abudefduf troschelii (Gill, 1862)	Adult and juvenile	Facultative	Eastern Pacific: Gulf of California; Bahía San Juanico, Baja California, Mexico to northern Peru, the Galapagos	McCourt and Thomson (1984)
	Chromis punctipinnis (Cooper, 1863)	Adult and juvenile	Facultative	Eastern Pacific: Monterey Bay, California (USA) to central Baja California (Mexico)	Hixon (1979)
	Microspathodon chrysurus (Cuvier, 1830)	Juvenile	Facultative	Western Atlantic: southern Florida (USA) and Bermuda through the Caribbean Sea to Brazil[37]	Randall (1958)

Table 2.1 cont.

	Microspathodon dorsalis (Gill, 1862)	Unreported	Facultative	Eastern Pacific: central Gulf of California to Malpelo Island (Colombia); Revillagigedo, Cocos, Galapagos Islands[38]	McCourt and Thomson (1984)
	Stegastes rocasensis (Emery, 1972)	Unreported	Facultative	Western Atlantic: Brazil	Sazima *et al*. (2010)
Serrasalmidae	*Serrasalmus marginatus* Valenciennes, 1837	Unreported	Facultative	South America: Paraguay-Paraná River basin	Sazima and Machado (1990)
Sparidae	*Diplodus argenteus argenteus* (Valenciennes, 1830)	Juvenile	Facultative	Western Atlantic: Southern Florida (USA), Bahamas, Antilles, and coast of South America[14]	Krajewski (2007)
	Diplodus holbrookii (Bean, 1878)	Juvenile	Facultative	Western Atlantic: Chesapeake Bay to Florida (USA); northeastern Gulf of Mexico	Carr and Adams (1972)
	Diplodus puntazzo (Walbaum, 1792)	Unreported	Facultative	Eastern Atlantic: Bay of Biscay to Sierra Leone, the Canary and Cape Verde Islands; Mediterranean, Strait of Gibraltar; Black Sea[39]; South Africa	Sazima *et al*. (2010)
	Oblada melanura (Linnaeus, 1758)	Unreported	Facultative	Eastern Atlantic: Mediterranean; Strait of Gibraltar to Angola; Madeira, Cape Verde and Canary Islands	Moosleitner (1980)
Syngnathidae	*Dunckerocampus baldwini* Herald & Randall, 1972	Adult	Facultative	Indo-Pacific: Christmas Island, Indonesia and Hawaii	Michael and Randall (1998)
	Dunckerocampus dactyliophorus (Bleeker, 1853)	Adults	Facultative	Indo-Pacific: Red Sea and East Africa to Samoa, north to Japan[22] to Australia	Kuiter (1996)
	Dunckerocampus pessuliferus Fowler, 1938	Adults	Facultative	Western Central Pacific: Sulade Islands in the Sulu Archipelago[40]; Australia[41]	Kuiter and Tonozuka (2001b)
	Doryrhamphus excisus Kaup 1856	Unreported	Facultative	Indo-Pacific and Eastern Pacific: Persian Gulf and East Africa to the west coast of the Americas	Bray and Thompson (2011)

Table 2.1 cont.

	Doryrhamphus janssi (Herald & Randall, 1972)	Adults	Facultative	Western Central Pacific: Gulf of Thailand to Solomon Islands, north to Philippines, south to Queensland (Australia); Belau and Truk, Micronesia	Kuiter and Tonozuka (2001b)
	Doryrhamphus japonicus Araga & Yoshino, 1975	Adults	Facultative	Western Pacific: Japan to Indonesia; Korea	Kuiter and Tonozuka (2001b)
	Entelurus aequoreus (Linnaeus, 1758)	Unreported	Facultative	Eastern Atlantic: Iceland and Norway to Azores; Baltic Sea	Potts (1973)
	Syngnathus acus Linnaeus, 1758	Unreported	Facultative	Eastern Atlantic; Mediterranean, Aegean and Black seas	Potts (1973)
	Syngnathus typhle Linnaeus, 1758	Unreported	Facultative	Eastern Atlantic: Vardø, Norway, Baltic Sea and the British Isles to Morocco; Mediterranean, Black and Sea of Azov	Potts (1973)
Terapontidae	*Rhyncopelates oxyrhynchus* (Temminck & Schlegel, 1842)	Juvenile	Facultative	Western Pacific: southern Japan to the Philippines	Shigeta *et al*. (2001)
Tetraodontidae	*Canthigaster capistrata* (Lowe, 1839)	Unreported	Facultative	Eastern Central Atlantic: Oceanic Islands	Quimbayo *et al*. (2012)
	Canthigaster jactator (Jenkins, 1901*)*	Adult	Facultative	Pacific Ocean: Hawaii	Losey *et al*. (1994)
	Canthigaster punctatissima (Günther, 1870)	Adult	Facultative	Eastern Central Pacific: Guaymas, Mexico to Panama; Galapagos Islands	McCourt and Thomson (1984)
Tripterygiidae	*Forsterygion lapillum* Hardy, 1989	Unreported	Facultative	Southwest Pacific: New Zealand	Clements (2003)
	Lepidonectes bimaculatus Allen & Robertson, 1992	Unreported	Facultative	Southeast Pacific: Malpelo Island, Colombia	Quimbayo *et al.* (2010)
	Notoclinops caerulepunctus Hardy, 1989	Unreported	Facultative	Southwest Pacific: New Zealand	Clements (2003)

Table 2.1 cont.

Notoclinops segmentatus (McCulloch & Phillipps, 1923)	Unreported	Facultative	Southwest Pacific: New Zealand	Clements (2003)

*Cleaners as defined in the current study under cleaning symbiosis; documented by cited authors; taxonomy updated. Exclusions: The cleaner "*Coris giofredi*" listed by Eibl-Eibesfeldt (1955) and cited in Feder (1966) is excluded here because the species is either an obscure synonym or is invalid; *Atherinops affinis* (Ayres, 1860) by Swartz (1981) because of insufficient evidence; *Remora albescens* (Temminck & Schlegel, 1850) excluded, cited by Côté (2000) from Cressey and Lachner (1970) who, through a lack of host-parasitic dietary evidence from stomach contents, indicated this species was not a cleaner; *Paramonacanthus oblongus* (Temminck & Schlegel, 1850) by Kearn (1978) because of insufficient evidence; *Pleuronectes schrenki* (Schmidt, 1904), by Ho *et al*. (2001) because of insufficient evidence; *Cyclopterus lumpus* because there is currently only evidence for this species interacting as an incidental cleaner; various taxa listed in the historic literature as "sp."; Synonyms, where *Crenilabrus scina* (Forsskål, 1775) = *Symphodus rostratus; Crenilabrus quinquemaculatus* Risso, 1827 = *Symphodus roissali; Doryrhamphus melanopleura* (Bleeker, 1858) = *D. excisus; Elacatinus inornatus* Bussing, 1990 = *E. digueti; Echeneis pallida* Temminck & Schlegel, 1850 = *Remora brachyptera*; *Labrus ossifagus* Linnaeus, 1758 and *Labrus bimaculatus* Linnaeus, 1758 = *L. mixtus*; *Pseudotropheus pursus* Stauffer, 1991 = *Maylandia pursa*; *Thalassoma duperry* = *T. dupperey*; *Thalassoma klunzingeri* Fowler & Steinitz, 1956 = *T. ruepellii, Xenestius californiensis* (incorrect spelling of *Xenistius californiensis* (Steindachner, 1876)) = *Haemulon californiensis*. **All distribution data are current and cited for the online database FishBase (Froese and Pauly 2015). ***This species is cited in Shigeta *et al*. (2001) from Kuwamura (1976) but is not mentioned in the latter. It may be an unintentionally produced typographical error where *Prionurus scalprum* Valenciennes, 1835 is supposed to be *P. microlepidotus*, the only cleaning acanthurid mentioned by Kuwamura (1976). [1]Desoutter (1990), [2]Randall (1986a), [3]Randall *et al.* (1990), [4]Sokolovskaya *et al.* (1998), [5]Cervigón (1993), [6]Berry and Smith-Vaniz (1978), [7]Randall *et al*. (1985), [8]Randall *et al.* (2003), [9]Floeter *et al*. (2003), [10]Lubbock and Edwards (1981), [11]van der Elst (1993), [12]Kottelat (2006), [13]Cervigón *et al.* (1992), [14]Smith (1997), [15]Sabaj and Ferraris (2003), [16]López *et al.* (2005), [17]Page and Burr (1991), [18]Page and Burr (2011), [19]Sazima *et al.* (2008), [20]Cervigón (1993), [21]Parenti and Randall (2000),

[22]Masuda *et al*. (1984), [23]Gomon (2006), [24]Afonso *et al.* (1999), [25]Robins and Ray (1986), [26]Lieske and Myers (1994), [27]Randall (1986c), [28]Tinker (1978), [29]Photographic record of 10 000 images of dead fishes cited in FishBase (Froese and Pauly 2015) as "Randall (1997)", [30]Myers (1999), [31]Sommer *et al*. (1996), [32]Myers (1991), [33]Heemstra (1984), [34]Fricke (1999), [35]Scott and Scott (1988), [36]Allen (1986), [37]De Moura *et al*. (1999), [38]Schneider and Krupp (1995), [39]Bauchot and Hureau (1986), [40]Dawson (1985), [41]Hoese *et al*. (2006).

Table 2.2. List of crustaceans currently considered as cleaners*.

Family (listed alphabetically)	Representative taxa	Primary cleaning activity period	Cleaning lifestyle	Distribution**	References
Alpheidae	*Alpheus djiboutensis* de Man, 1909	Diurnal[1]	Facultative	Red Sea[2]	Karplus *et al.* (1972)
	Alpheus randalli Banner & Banner, 1980	Diurnal[1]	Facultative	Indo-Pacific	Karplus (2014)
	Alpheus rapax Fabricius, 1798	Unreported	Facultative	Indo-Pacific	Hou *et al.* (2013)
Gnathophyllidae	*Gnathophyllum panamense* Faxon, 1893	Unreported	Facultative	Guaymas, Sonoro (Mexico)[3]	McCourt and Thomson (1984)
Hippolytidae	*Lysmata amboinensis* (de Man, 1888)[§]	Diurnal[1]	Dedicated	Indo-Pacific[4]	Baensch and Debelius (1992); Côté (2000)
	Lysmata ankeri Rhyne & Lin, 2006[§]	Unreported	Facultative	Caribbean[5]	Baeza (2009)
	Lysmata argentopunctata Wicksten, 2000	Unreported	Facultative	Gulf of California to Costa Rica and Ecuador (including Galapagos Islands)[6]	Wicksten (2009)
	Lysmata bahia Rhyne & Lin, 2006[§]	Unreported	Facultative	Western Atlantic[5]	Baeza (2009)
	Lysmata boggessi Rhyne & Lin, 2006[§]	Unreported	Facultative	Caribbean[6]	Baeza (2009)
	Lysmata californica (Stimpson, 1866)	Nocturnal	Facultative	Santa Barbra, California (USA) to Baja California, Guadalupe Island (Mexico)	Limbaugh *et al.* (1961)
	Lysmata debelius Bruce, 1983	Diurnal[1]	Dedicated	Indo-Pacific[4]	Baensch and Debelius (1992); Côté (2000)

Table 2.2. cont.

Lysmata galapagensis Schmitt, 1924	Unreported	Facultative	Galapagos and tropical west Pacific[4]	Baensch and Debelius (1992); Côté (2000)
Lysmata grabhami (Gordon, 1935) §	Diurnal[1,7]	Dedicated	West, Central, East equatorial Atlantic; Gulf of Mexico; Madeira	Limbaugh *et al.* (1961)
Lysmata gracilirostris Wicksten, 2000	Unreported	Facultative	Eastern tropical Pacific[5]	Baeza (2009)
Lysmata hochi Baeza & Anker, 2008	Unreported	Facultative	Caribbean[5]	Baeza (2009)
Lysmata intermedia (Kingsley, 1878) §	Unreported	Facultative	Azores; Ascensión; eastern Pacific; western Atlantic	Baeza (2009)
Lysmata kuekenthali (de Man, 1902)	Unreported	Unreported	Indian Ocean[4]	Baensch and Debelius (1992); Côté (2000)
Lysmata moorei (Rathbun, 1901)	Unreported	Facultative	Caribbean; western Atlantic[5]	Baeza (2009)
Lysmata nayaritensis Wicksten, 2000	Unreported	Facultative	Eastern Pacific[5]	Baeza (2009)
Lysmata pederseni Rhyne & Lin, 2006§	Unreported	Facultative	Caribbean[5]	Baeza (2009)
Lysmata rafa Rhyne & Anker, 2007	Unreported	Facultative	Caribbean[5]	Baeza (2009)
Lysmata rathbunae Chace, 1970§	Unreported	Unreported	Australia[4]	Baensch and Debelius (1992); Côté (2000)
Lysmata seticaudata (Risso, 1816) §	Nocturnal[1]	Facultative	Mediterranean[4]; Azores[8]	Moosleitner (1980)
Lysmata splendida Burukovsky, 2000	Unreported	Dedicated	Maldives[9]	Karplus (2014)

Table 2.2. cont.

	Lysmata ternatensis de Man, 1902	Unreported	Unreported	Indo-Pacific[1]	Debelius (1999); Karplus (2014)
	Lysmata vittata (Stimpson, 1860)	Unreported	Facultative	Western Indian and western Pacific Oceans	Baensch and Debelius (1992); Côté (2000)
	Lysmata wurdemanni (Gibbes, 1850) [§]	Nocturnal[1]	Facultative	Gulf of Mexico[5]; eastern Atlantic[5]; Caribbean[5]	Baeza (2009)
	Parhippolyte uveae Borradaile, 1900	Unreported	Unreported	Indo-Pacific[4]	Baensch and Debelius (1992); Côté (2000)
Palaemonidae	*Ancylomenes adularans* (Bruce, 2003)	Unreported	Unreported	Australia[1]; southern Japan[1]; Taiwan[1]	Okuno (2005); Okuno and Bruce (2010)
	Ancylomenes aesopius (Spence Bate, 1863)	Unreported	Unreported	South and Western Australia[11]	Shepherd *et al.* (2005)
	Ancylomenes anthophilus (Holthuis & Eibl-Eibesfeldt, 1964)***, §	Diurnal[1]	Unreported	Whalebone Bay, Bermuda[11]	Okuno and Bruce (2010)
	Ancylomenes holthuisi (Bruce, 1969) [§]	Unreported	Unreported	Indo-Pacific	Becker and Grutter (2004)
	Ancylomenes kobayashii (Okuno & Nomura, 2002)	Unreported	Unreported	Japan[11]	Okuno and Bruce (2010)
	Ancylomenes longicarpus (Bruce & Svoboda, 1983)	Crepuscular	Dedicated	Red Sea and Arabian Peninsula[12]	Chapuis and Bshary (2009)
	Ancylomenes lucasi (Chace, 1937)	Unreported	Unreported	Guaymas, Sonoro (Mexico)[3]	McCourt and Thomson (1984)
	Ancylomenes magnificus (Bruce, 1979)	Unreported	Unreported	Indo-Pacific	Becker *et al.* (2005)

Table 2.2. cont.

Ancylomenes pedersoni (Chace, 1958)[§]	Diurnal[1]	Dedicated	West Indies[13]	Limbaugh *et al.* (1961)
Ancylomenes speciosus (Okuno, 2004)	Unreported	Unreported	Japan[11]; Ogasawara Islands, east China Sea[11]; Australia[11]; New Caledonia[11]	Okuno and Bruce (2010)
Brachycarpus biunguiculatus (Lucas, 1846)	Nocturnal	Unreported	Mediterranean Sea; Red Sea; Sri Lanka; Ascensión, Bermuda, Hawaiian Islands; subtropical Atlantic; eastern Pacific	Corredor (1978)
Palaemon adspersus Rathke, 1837	Unreported	Facultative	Algeria; Baltic, Black, Caspian, Mediterranean Seas; Egypt; India; Libya; southern Norway; Spain; British Isles; eastern Atlantic	Östlund-Nilsson *et al*. (2005)
Palaemon elegans Rathke, 1837	Diurnal[1]	Facultative	Northern, eastern Atlantic; Aral, Baltic, Black, Caspian, Mediterranean, Red Seas	Östlund-Nilsson *et al*. (2005)
Palaemon ritteri Holmes, 1895	Unreported	Facultative	Puerto Peñasco, Sonora (Mexico)[3]; Gulf of Mexico[4]	McCourt and Thomson (1984)
Periclimenes yucatanicus (Ives, 1891)	Unreported	Dedicated	Florida (USA) to Colombia[14]; West Indies[14]; Puerto Rico[14]	Limbaugh *et al.* (1961)
Urocaridella antonbruunii (Bruce, 1967)	Unreported	Dedicated	Western Indian and Pacific Oceans; Levantine Sea	Corredor (1978)

Table 2.2. cont.

	Urocaridella pulchella Yokes & Galil, 2006	Nocturnal	Facultative	Mediterranean, coast of Turkey[15]	Yokes and Galil (2006)
Pandalidae	*Plesionika longicauda* (Rathbun, 1901)	Unreported	Unreported	Eastern Atlantic; South Africa	Jonasson (1987)
Stenopodidae	*Stenopus hispidus* (Olivier, 1811)	Nocturnal	Facultative	Circumtropical; Durban (South Africa); Mozambique	Corredor (1978)
	Stenopus pyrsonotus Goy & Devaney, 1980	Nocturnal[1]	Unreported	Indo-West Pacific[16]	Debelius (1999); Calado (2008)
	Stenopus scutellatus Rankin, 1898	Nocturnal	Unreported	Gulf of Mexico[17]	Corredor (1978)
	Stenopus spinosus Risso, 1827	Crepuscular/nocturnal[18]	Unreported	Southeastern Atlantic; Mediterranean[17]	Wood (2015)
	Stenopus tenuirostris de Man, 1888	Unreported	Unreported	Indo-Pacific[19]	Holthuis (1946)

*Cleaners as defined in the current study; documented by cited authors; taxonomy updated according to the online database WoRMS. Exclusions: *Stenopus chrysexanthus* Goy, 1992 and *S. cyanoscelis* Goy, 1992 – suggested to be cleaners based on morphology (Goy 1992) but without any observation or other evidence; *Stenopus zanzibaricus* Bruce, 1976 – anecdotal record of Greenley (2013); "*Lysmata striata"* (see Baeza 2009) could not be confirmed as a valid taxon (Anker and Cox 2011; Boxshall *et al*. 2016) and could be an obscure synonym for *L. seticaudada* where *Aglaope striata* Rafinesque, 1814 = *L. striata*; *Barbouria cubensis* (von Martens, 1872), *Eualus cranchii* (Leach, 1817 [in Leach, 1815-1875]), *E. occultus* (Lebour, 1936), *Hippolyte inermis* Leach, 1816, *H. nicholsoni* Chace, 1972, *H. prideauxiana* Leach, 1817 [in Leach, 1815-1875], *H.*

varians Leach, 1814 [in Leach, 1813-1814], *Lebbeus groenlandicus* (Fabricius, 1775), *L. polaris* (Sabine, 1824), *Lysmata multiscissa* (Nobili, 1904), *L. nilita* Dohrn & Holthuis, 1950, *L. zacae* Armstrong, 1941, *Periclimenes imperator* Bruce, 1967, *Thor amboinensis* (de Man, 1888), *Tozeuma carolinense* Kingsley, 1878 – no supporting data (Debelius 1999); *Odontozona* sp. (Goy 2010) (= *O. rubra* Wicksten, 1982, pers comm. J. W Goy) – no supporting data; *Periclimenes paivai* Chace, 1969 – no supporting data (Martinelli Filho *et al.* 2008); *Planes minutus* (Linnaeus, 1758), *Stenorhynchus lanceolatus* (Brullé, 1837) – insufficient data; Any unidentified *Ancylomenes* or *Periclimenes* spp.; "*Urocaridella* spp. a, b, c" – undescribed (Okuno 1994; Debelius 1999); Synonyms, where *Leandrites cyrtorhynchus* Fujino & Miyake, 1969 = *Urocaridella antonbruunii; Parapandulus longicauda* (invalid) = *Plesionika longicauda; Periclimenes pedersoni* Chace, 1958 = *Ancylomenes pedersoni; P. holthuisi* Bruce, 1969 = *A. holthuisi; P. longicarpus* Bruce & Svoboda, 1983 = *A. longicarpus; P. lucasi* Chace, 1937 = *A. lucasi.* **Distribution data are cited for the online database WoRMS (only data verified by WoRMS taxonomic editors considered; Boxshall *et al*. 2016); [1]Karplus (2014); [2]Karplus *et al.* (1972); [3]McCourt and Thomson (1984); [4]Côté (2000); [5]Baeza (2009); [6]Anker and Cox (2011); [7]Criales and Corredor (1977); [8]d'Udekem d'Acoz (2000); [9]Burukovsky (2000); [10]Bruce (2011); [11]Okuno and Bruce (2010); [12]Chapuis and Bshary (2009); [13]Ochoa (2015); [14]Limbaugh *et al*. (1961); [15]Yokes and Galil (2006); [16]Goy and Devaney (1980); [17]Lukens (1977); [18]Wood (2015); [19]Calado *et al.* (2003); [20]Bruce (1976). ***The new taxonomic combination of Okuno and Bruce (2010) considered, to the exclusion of the synonym proposed by Wicksten (1995) and Spotte (1999) for *A. pedersoni.* [§]Species that form part of a species complex or have been historically confused or misidentified (see Baeza 2010; Baeza and Anker 2008; Debelius 1999; d'Udekem d'Acoz 2000; Hayashi 1975; Okuno and Bruce 2010; Rhyne and Lin 2006).

Table 2.3. Ectoparasite or epibiont categories and their nutritional source, from the gut contents of wild cleaners, or removed and consumed by captive cleaners.

Cleaner	Ectoparasite or epibiont food item (after source)*	Category	Parasite nutrition source[1]	Wild (W) or captive (C)	References
Ancylomenes holthuisi	*Benedenia* sp.	Monogenean parasites	Mucus	C	Becker and Grutter (2004)
	Copepoda	Crustacean parasites	Skin or mucus	W, C	
	Gnathiidae	Crustacean parasites	Blood	W, C	
Ancylomenes pedersoni	*Anilocra haemuli* Williams & Williams, 1981	Crustacean parasites	Blood	C	Bunkley-Williams and Williams (1998)
	Neobenedenia melleni (MacCallum, 1927) Yamaguti, 1963	Monogenean parasites	Mucus	C	McCammon *et al*. (2010)
Apeltes quadracus	*Gyrodactylus* sp.	Monogenean parasites	Mucus	C	Tyler (1963)
Atypichthys strigatus	Ectoparasitic copepods (Caligoida and Monstriloida)	Crustacean parasites	Skin or mucus	W	Glasby and Kingsford (1994)
Canthidermis maculata	Parasitic isopod	Crustacean parasites	Blood	W	Gooding (1964)
Centrolabrus exoletus	*Caligus coryphaenae* Steenstrup & Lütken, 1861	Crustacean parasites	Skin or mucus	C	Tully *et al.* (1996)
	Caligus sp.	Crustacean parasites	Skin or mucus	W	Galeote and Otero (1998)
	Calliobdella lophii Van Beneden & Hesse, 1863	Hirudinean parasites	Blood	C	Samuelsen (1981)
	Gnathia sp.	Crustacean parasites	Blood	W	Galeote and Otero (1998)
	Hatschekia sp.	Crustacean parasites	Skin or mucus	W	

Table 2.3. cont.

	Lepeophtheirus salmonis (Krøyer, 1837)	Crustacean parasites	Skin or mucus	C	Bjordal (1988)
Chaetodon citrinellus	Gnathiidae	Crustacean parasites	Blood	W	Grutter (2002)
Chaetodon kleinii	*Amyloodinium ocellatum* (E. Brown) E. Brown & Hovasse, 1946	Dinoflagellate parasites	Host epithelial tissues[2]	C	Present study
Coris julis	Isopod	Crustacean parasites	Blood	W	Van Tassell *et al.* (1994)
Coris sandageri	*Codonophilus* sp.	Crustacean parasites	Blood	W	Ayling and Grace (1971)
Ctenolabrus rupestris	*Caligus coryphaenae*	Crustacean parasites	Skin or mucus	C	Tully *et al.* (1996)
	Gnathia maxillaris (Montagu, 1804)	Crustacean parasites	Blood	C	Potts (1973)
	Lepeophtheirus salmonis	Crustacean parasites	Skin or mucus	C	Bjordal (1988)
Cyprinodon variegatus	Leech (unidentified)	Hirudinean parasites	Blood	C	Able (1976)
Diplodus holbrookii	*Argulus* sp.	Crustacean parasites	Skin or mucus	W	Carr and Adams (1972)
Echeneis naucrates	*Alebion carchariae* Krøyer, 1863	Crustacean parasites	Skin or mucus	W	Cressey and Lachner (1970)
	Argulus sp.	Crustacean parasites	Skin or mucus	W	
	Caligus sp.	Crustacean parasites	Skin or mucus	W	
	Isopods (larval)	Crustacean parasites	Blood	W	
	Pandarid copepods (immature)	Crustacean parasites	Skin or mucus	W	
	Paralebion pearsei Causey, 1953	Crustacean parasites	Skin or mucus	W	
Elacatinus evelynae	*Bomolochus* sp.	Crustacean parasites	Skin or mucus	W	Whiteman and Côté (2002)
	Caligus sp.	Crustacean parasites	Skin or mucus	W	
	Cymothoid isopods	Crustacean parasites	Blood	W	Losey (1974)

Table 2.3. cont.

	Gnathiid isopods	Crustacean parasites	Blood	W	
	Gnathiid larvae	Crustacean parasites	Blood	W	Whiteman and Côté (2002)
Elacatinus figaro	*Neobenedenia melleni*	Monogenean parasites	Mucus	C	de Souza *et al*. (2014)
Elacatinus genie	Cymothoid isopods	Crustacean parasites	Blood	W	Losey (1974)
	Gnathiid isopods	Crustacean parasites	Blood	W	
	Neobenedenia melleni	Monogenean parasites	Mucus	C	Cowell *et al*. (1993)
Elacatinus oceanops	*Neobenedenia melleni*	Monogenean parasites	Mucus	C	Cowell *et al*. (1993)
Elacatinus prochilos	Caligidae	Crustacean parasites	Skin or mucus	W	Arnal and Côté (2000)
	Gnathiid isopods	Crustacean parasites	Blood	W	
Entelurus aequoreus	Caligoid copepods	Crustacean parasites	Skin or mucus	C	Potts (1973)
	Gnathiid larvae	Crustacean parasites	Blood	C	
Girella nigricans	Calagoid copepods	Crustacean parasites	Skin or mucus	W	DeMartini and Coyer (1981)
Halichoeres cyanocephalus	Gnathiid larvae	Crustacean parasites	Blood	W	Sazima *et al*. (1998b)
Heniochus monoceros	Gnathiidae	Crustacean parasites	Blood	W	Grutter (2002)
Hermosilla azurea	Calagoid copepods	Crustacean parasites	Skin or mucus	W	DeMartini and Coyer (1981)
Labroides bicolor	Calagoid copepods	Crustacean parasites	Skin or mucus	W	Randall (1958)
	Gnathiid isopods	Crustacean parasites	Blood	W	
Labroides dimidiatus	*Benedenia lolo* Yamaguti, 1968	Monogenean parasites	Mucus	C	Grutter *et al.* (2002)
	Bomolochid copepods	Crustacean parasites	Skin or mucus	W	Grutter (1995)
	Calagoid copepods	Crustacean parasites	Skin or mucus	W	Randall (1958)
	Calagid copepods	Crustacean parasites	Skin or mucus	W	
	Cryptocaryon irritans Brown, 1951	Ciliophoran parasites	Host epithelial tissues	C	Grutter (2002)
	Dissonus sp.	Crustacean parasites	Skin or mucus	W	Gorlick *et al*. (1987)

Table 2.3. cont.

	Gnathiid isopods	Crustacean parasites	Blood	W	Grutter (1996b)
	Hatschekia sp.	Crustacean parasites	Skin or mucus	W	Grutter (1995)
	Lernaeid copepod	Crustacean parasites	Skin or mucus	W	Randall (1958)
	Monogeneans	Monogenean parasites	Mucus	C	Grutter and Bshary (2003)
	Penellid copepods	Crustacean parasites	Skin or mucus	W	Randall (1958)
	Trematode[3]	Monogenean parasites	Mucus	W	Gorlick *et al*. (1987)
Labroides phthirophagus	Calagoid copepods	Crustacean parasites	Skin or mucus	W	Randall (1958)
	Gnathiid isopods	Crustacean parasites	Blood	W	
	Lernaeid copepod	Crustacean parasites	Skin or mucus	W	
Labroides rubrolabiatus	Gnathiid isopods	Crustacean parasites	Blood	W	Randall (1958)
Labrus bergylta	*Lepeophtheirus salmonis*	Crustacean parasites	Skin or mucus	C	Leclercq *et al.* (2014)
Labrus mixtus	*Lepeophtheirus salmonis*	Crustacean parasites	Skin or mucus	C	Bjordal (1991)
Lepadogaster candolii	Gnathiidae	Crustacean parasites	Blood	W	Weitzmann and Mercader (2012)
Lepomis macrochirus	*Argulus* sp.	Crustacean parasites	Skin or mucus	C	Spall (1970)
Lepomis megalotis	*Argulus* sp.	Crustacean parasites	Skin or mucus	C	Spall (1970)
Lysmata amboinensis	*Neobenedenia* sp.	Monogenean parasites	Mucus	C	Militz and Hutson (2015)
Oligoplites saurus	*Argulus* sp.	Crustacean parasites	Skin or mucus	W	Carr and Adams (1972)
	Caligoid copepods	Crustacean parasites	Skin or mucus	W	Lucas and Benkert (1983)
Oxyjulis californica	*Caligus hobsoni* Cressey, 1969	Crustacean parasites	Skin or mucus	W	Hobson (1971)
	Caligus serratus Shiino, 1965	Crustacean parasites	Skin or mucus	W	
	Gnathiid isopods	Crustacean parasites	Blood	W	
	Lepeophtheirus sp.	Crustacean parasites	Skin or mucus	W	
Palaemon adspersus	*Gyrodactylus* sp.	Monogenean parasites	Mucus	C	Östlund-Nilsson *et al*. (2005)

Table 2.3. cont.

	Lepeophtheirus pectoralis (Müller O.F., 1776)	Crustacean parasites	Skin or mucus	C	
Palaemon elegans	*Gyrodactylus* sp.	Monogenean parasites	Mucus	C	Östlund-Nilsson *et al*. (2005)
Periclimenes yucatanicus	*Neobenedenia melleni*	Monogenean parasites	Mucus	C	McCammon *et al*. (2010)
Phanerodon atripes	Caligid copepods	Crustacean parasites	Skin or mucus	W	Hobson (1969)
Pomoxis annularis	*Argulus* sp.	Crustacean parasites	Skin or mucus	W	Spall (1970)
Pseudotropheus crabro	*Argulus africanus* Thiele, 1900	Crustacean parasites	Skin or mucus	W	Ribbink and Lewis (1981)
Remora brachyptera	Copepods (immature)	Crustacean parasites	Skin or mucus	W	Cressey and Lachner (1970)
	Caligid copepod (immature)	Crustacean parasites	Skin or mucus	W	
	Gloiopotes huttoni (Thomson G.M., 1890)	Crustacean parasites	Skin or mucus	W	
	Gloiopotes watsoni Kirtisinghe, 1934	Crustacean parasites	Skin or mucus	W	
	Phyllothyreus cornutus (Milne Edwards, 1840)	Crustacean parasites	Skin or mucus	W	
Remora osteochir	Caligoid copepod	Crustacean parasites	Skin or mucus	W	Cressey and Lachner (1970)
	Caligus sp.	Crustacean parasites	Skin or mucus	W	
	Gloiopotes americanus Cressey, 1967	Crustacean parasites	Skin or mucus	W	
	Gloiopotes ornatus Wilson C.B., 1905	Crustacean parasites	Skin or mucus	W	
	Pennella sp.	Crustacean parasites	Skin or mucus	W	

Table 2.3. cont.

Remora remora	*Achtheinus dentatus* Wilson C.B., 1911	Crustacean parasites	Skin or mucus	W	Szidat and Nani (1951)
	Alebion carchariae Krøyer, 1863	Crustacean parasites	Skin or mucus	W	Cressey and Lachner (1970)
	Copepods (immature)	Crustacean parasites	Skin or mucus	W	
	Echthrogaleus coleoptratus (Guérin-Méneville, 1837)	Crustacean parasites	Skin or mucus	W	
	Gangliopus pyriformis Gerstaecker, 1854	Crustacean parasites	Skin or mucus	W	
	Pandarus cranchii Leach, 1819	Crustacean parasites	Skin or mucus	W	Cressey and Lachner (1970)
	Pandarus satyrus Dana, 1849	Crustacean parasites	Skin or mucus	W	
	Pennella sp.	Crustacean parasites	Skin or mucus	W	
	Phyllothyreus cornutus	Crustacean parasites	Skin or mucus	W	
Rhyncopelates oxyrhynchus	Caligid copepods	Crustacean parasites	Skin or mucus	W	Shigeta *et al* (2001)
Serrasalmus marginatus	*Dolops* spp.	Crustacean parasites	Skin or mucus	W	Sazima and Machado (1990)
Stenopus hispidus	*Neobenedenia melleni*	Monogenean parasites	Mucus	C	McCammon *et al*. (2010)
Symphodus melanocercus	*Caligus* spp.	Crustacean parasites	Skin or mucus	W	Arnal and Morand (2001)
	Gnathiid isopods	Crustacean parasites	Blood	W	Flückiger (1981)
	Peniculus fistula fistula Nordmann, 1832	Crustacean parasites	Skin or mucus	W	Arnal and Morand (2001)
Symphodus melops	*Gnathia maxillaris*	Crustacean parasites	Blood	C	Potts (1973)
	Lepeophtheirus salmonis	Crustacean parasites	Skin or mucus	C	Bjordal (1988)
Syngnathus acus	Caligoid copepods	Crustacean parasites	Skin or mucus	C	Potts (1973)

Table 2.3. cont.

	Gnathiid larvae	Crustacean parasites	Blood	C	
Syngnathus typhle	Caligoid copepods	Crustacean parasites	Skin or mucus	C	Potts (1973)
	Gnathiid larvae	Crustacean parasites	Blood	C	
Thalassoma bifasciatum	Calagid copepods	Crustacean parasites	Skin or mucus	W	Losey (1974)
	Cymothoid isopods	Crustacean parasites	Blood	W	Losey (1974)
	Gnathiid isopods	Crustacean parasites	Blood	W	Losey (1974)
	Lernaeid copepods	Crustacean parasites	Blood	W	Losey (1974)
	Neobenedenia melleni	Monogenean parasites	Mucus	C	Cowell *et al*. (1993)
Thalassoma duperrey	*Platylepas hexastylos* (Fabricius, 1798)	Crustacean commensals	Plankton	W	Losey *et al.* (1974)
Urocaridella sp. c	*Benedenia* sp.	Monogenean parasites	Mucus	C	Becker and Grutter (2004, 2005)
	Copepoda	Crustacean parasites	Skin or mucus	W	Becker and Grutter (2004)
	Gnathiidae	Crustacean parasites	Blood	W	Becker and Grutter (2004)

[1]Parasite nutritional source generally considered for respective groups based on Rohde (2005), or [2]Noga and Levy (2006). [3]"Trematode" considered here as the historic inclusive taxonomic term use at that time for 'monogenean', because it was listed specifically as a prey item by Gorlick *et al.* (1987), and would have been removed from the external surface of the host fish. *Synonyms*: Caligus elongatus* Heegaard, 1943 = *C. coryphaenae*; *Pandarus armatus* Heller, 1865 = *P. cranchii*; *Phyllothereus cornutus* (invalid) = *Phyllothyreus cornutus;* Excluded: *Midias lobates* (from Cressey and Lachner 1970) = unknown taxon.

CHAPTER 3

Cleaner shrimp are true cleaners of injured fish

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In this chapter, I investigate the ability of cleaner shrimp to tend to injured fish through true symbiotic cleaning interactions, to address the second thesis aim.

3.1 Abstract

Reef fishes sustain injuries from various behavioural and environmental interactions. Injured fishes have been observed frequenting cleaning stations to be attended by different cleaner fishes. This symbiotic relationship between injured fishes and cleaner fishes has only been observed in the wild, and has never been demonstrated empirically for cleaner shrimp. The first investigation of cleaning of injured fish by cleaner shrimp, and the first controlled laboratory trial investigating the functional relationship between injured client fish and cleaners, is

presented. I tested whether the cleaner shrimp (*Lysmata amboinensis*) cleaned injured sea goldies (*Pseudanthias squamipinnis*) following a standardised, unilateral superficial skin lesion. I recorded the cleaning behaviour between shrimp and fish and determined that the fish regulated the cleaning, and reduced the amount of cleaning time by the shrimp of the injured side immediately post-injury, corresponding with previous literary evidence of the rapid onset of re-epithelialisation of the injury to seal it in the first 24 hours in injured fishes. Thereafter, injured fish showed no cleaning preference between injured and uninjured sides. Image analyses determined that the cleaner shrimp reduced the redness of the injury, representing rubor, associated with the inflammatory response in fishes. Injuries in fishes are susceptible to invasion by secondary pathogens, and the reduction of injury rubor by shrimp may suggest that cleaning by these shrimp could reduce the success of opportunistic infection. Cleaner shrimp neither aggravated existing injury, nor created additional injury, measured quantitatively. The cleaning of injured fish by cleaner shrimp thus likely involves true cleaning behaviour.

3.2 Introduction

The ecological significance of cleaning has been demonstrated since the early 1960s (see Chapter 2). Cleaning behaviour between cleaner and client organisms is mutually beneficial (Côté 2000). Clients benefit by the reduction of negative effects associated with dead or damaged tissue, ectoparasite or epibiont loads, which serve as an important food source for cleaners (Losey *et al*. 1994; Sazima *et al.* 2004a, Rohde 2005; Sazima *et al.* 2010, 2012; Chapter 2). The removal of cleaner fishes from patch reefs where they service clients, impacts not only the number and diversity of fishes that visit (Limbaugh 1961; Bshary 2003; Grutter *et al.* 2003; Waldie *et al*. 2011), or recruit as juveniles to these reefs (Waldie *et al.* 2011; Sun *et al*. 2015), but also results in an increase in visible client lesions (Limbaugh 1961) and ectoparasite infestations (Grutter *et al.* 2017). Therefore, cleaner fishes have an important role

to play in the health and community structure of other reef fishes. Similar studies have not been performed to demonstrate the impact that the removal of cleaner shrimp might have on reef fishes, largely because of the constraints in doing so in the wild. Many shrimp are nocturnal, often cryptic and crevice-dwelling, and cannot simply or reliably be removed from a patch reef (Vaughan *et al*. 2016; Chapter 2).

Reef fishes naturally incur injuries sustained from various behavioural interactions (Kolm *et al.* 2005), parasites (Purivirojkul 2012), and environmental events (Walsh 1983). Apart from visible lesions, fishes may also exhibit a change in epithelial pigmentation as a response to stress or injury (Iger *et al*. 1995; Militz and Hutson 2015). The cleaning of wounds on injured reef fishes by cleaner organisms has been documented infrequently in the literature. These few accounts include the incidental observations discussed briefly by Limbaugh (1961), and Hobson (1971), and the detailed observations of a single 18 month study of injured Caribbean reef fishes by Foster (1985). Notably, injured fishes spent longer periods visiting cleaner fishes than after their wounds had healed. Foster's (1985) work suggested that injury could be a proximate cause of cleaning behaviour in fishes. However, the most commonly explored and reported proximate causes of cleaning in the literature include ectoparasite loads and epibiont burdens of client organisms (e.g. Losey *et al.* 1994; Arnal and Morand 2001; Grutter 2001; Sazima *et al.* 2004a; Sikkel *et al.* 2004; Bertoncini *et al*. 2009; Sazima *et al.* 2010; Grutter *et al*. 2017). Cleaning behaviour between cleaner and client organisms is mutually beneficial (Côté 2000). Clients benefit by the reduction of negative effects associated with the dead or damaged tissue, ectoparasite or epibiont loads, which serve as an important food source for cleaners (Losey *et al.* 1994; Sazima *et al.* 2004a; Rohde 2005; Sazima *et al.* 2010, 2012).

Research on cleaner fishes has traditionally dominated the literature, and comparatively few articles on cleaner shrimp have been published (Titus *et al.* 2015; Vaughan *et al.* 2016;

Chapter 2). Historically, it was assumed that cleaner shrimp feed on the ectoparasites of fishes, but only five studies have actually demonstrated the ability of cleaner shrimp to remove and to consume fish ectoparasites, either directly off the client fish (Bunkley-Williams and Williams 1998; Becker and Grutter 2004; Östlund-Nilsson *et al.* 2005; McCammon *et al.* 2010), or environmentally (Militz and Hutson 2015). The cleaning of wounds by cleaner shrimp would have a similar ecologically significant function to that demonstrated by cleaner fish if it could be shown that injured fishes derive health benefits from such interactions. This evidence is currently lacking for cleaner shrimp (Vaughan *et al*. 2016; Chapter 2). To test the hypothesis that cleaner shrimp aid wound healing, the alternative possibility that cleaner shrimp might actually be taking advantage of an injured client; a temporary parasitic function synonymous with cheating (see Grutter 1997; Grutter and Bshary 2003), needs to be explored.

Cheating is a temporary deviation from the normal symbiotic relationship of many different mutualisms, and is represented by a brief exploitation of benefits by one partner of another, with reduced or no reciprocal benefits afforded in return (Ferreire *et al.* 2001). Examples of cheating in cleaning symbioses have been well documented, including examples of cleaners feeding on client mucus, and cleaners being eaten by their clients (e.g. Francili-Filho *et al.* 2000; Arnal *et al.* 2001; Cheney and Côté 2005). Certainly, cleaner shrimp are thought to cheat (Chapuis and Bshary 2009), and it is logical to conclude that cheating by shrimp would result in exploitation of an existing injury as a potential food source. This exploitation would result in sustained tissue damage from cheating shrimp, and would negatively impact the healing response and healing time. This is analogous to the exploitation of wounds by the Red-billed oxpecker birds in terrestrial cleaning interactions (see Weeks 2000). Therefore, to evaluate the nature of cleaning interactions between wounded fish and cleaner shrimp, it is crucial to be able to quantify the area of injured tissue, and to use

appropriate controls. In this chapter I aimed to test the hypothesis that cleaner shrimp are true cleaners of the wounds of injured fish, and not merely exploiting injured clients.

3.3 Methods

3.3.1 Statement on the welfare of animals

A superficial, standardised epithelial injury was necessary to answer the research question. Fish were given a single unilateral superficial injury by removal of scales from a predetermined area on either flank of an area no larger than 0.15 cm². All fish were housed separately to avoid bullying. Handling of all fish briefly with an aquarium hand-held net was crucial for the transferral into and out of the experimental tanks and could not be avoided. Hand-netting was kept to the operational minimum, occurring only once per day in and out of the experimental tanks per individual. Fish were offered food daily after experimentation and fed successfully, demonstrating a rapid return to normal behaviour after brief handling. Anaesthesia was justifiably employed to facilitate rapid photography of all fish and to avoid prolonged aerial exposure. Three fish mortalities occurred during the experimentation period employing 126 fish. All fish were humanely euthanased after sampling using an anaesthetic overdose of 2-phenoxyethanol (1.5 ml/L >10 min; Neiffer and Stamper 2009) following the approved animal end-point set out in the animal ethics approval. This article does not contain any studies with human participants performed by any of the authors.

3.3.2 Animal maintenance

Experimental animal species were selected based on their successful use in prior studies (see Militz and Hutson 2015), their known natural relationship in a cleaning symbiosis, their small size, bright colour, captivity compatibility and commercial availability. One hundred and twenty-six wild-caught adult female sea goldies, *Pseudanthias squamipinnis* (Peters, 1885)

(10.07 ± 1.12 cm total length) and 29 adult cleaner shrimp, *Lysmata amboinensis* (de Man, 1888) (~6–7 cm long) were purchased from an approved commercial ornamental fish supplier, sourced from Australia. Adult *P. squamipinnis* are sexually dimorphic (Shapiro 1988) and known to interact with *L. amboinensis* in a cleaning symbiosis (see Militz and Hutson 2015). Therefore, to standardise for body colouration for photographic sampling and analyses, only female *P. squamipinnis* were purchased and used. All fish were given a five minute freshwater bath on arrival to the laboratory to rid them of potential ectoparasites (Kaneko *et al*. 1988; Hutson *et al.* 2018). Thereafter, they were quarantined in three identical 70 L plastic tanks connected to a dedicated recirculating marine life-support system for a minimum of three weeks. During the quarantine period, the fish were weaned from commercially available defrosted *Mysis* sp. shrimp and live *Artemia* Leach, 1819 sp. nauplii onto a high-density gel-based diet made by saturating 200 ml molten food-grade gelatin solution (19 g per 100 ml boiling freshwater) with Primo Aquaculture NRD 3/5 commercial ornamental fish broodstock diet (Ridley Agriproducts, Australia), refrigerating to set, and were fed to satiation twice daily. This was done to accommodate the high energy nutritional demand of this species, and to avoid nutritional deficiencies which may influence health. Cleaner shrimp were quarantined for three weeks separately in the laboratory, and held individually in identical 3 L labelled polyethylene aquaria with fitted lids coupled to a separate recirculating marine life-support system and fed daily with defrosted *Mysis* Latreille, 1802 sp. shrimp. Water quality parameters were maintained at (25 ± 1° C; 35 ppt, 8.1 pH and 86-96% DO saturation) for both fish and shrimp systems.

3.3.3 Inflicting wounds

A controlled, superficial injury given to the fish under anaesthetic, was necessary to record the influence of injury on interactive behaviour. The anaesthetic tricaine methanesulfonate (MS-

222), used by Davis and Ottmar (2006) had a negative effect on sodium fluorescein staining in their results (see also Davis *et al*. 2008). I avoided this by using the anaesthetic 2-phenoxyethanol (Sigma-Aldrich, Australia), an alternative fish anaesthetic that has a history of success in the anaesthesia of many teleosts and elasmobranchs (Vaughan *et al.* 2008; Penning *et al.* 2017). Prior to the experimentation, a pilot study was carried out to test the influence of 2-phenoxyethanol on sodium fluorescein staining of damaged fish epithelium. The anaesthetic 2-phenoxyethanol did not interfere with sodium fluorescein (Sigma-Aldrich, Australia) staining at the concentration used in the methods. At the commencement of the experiment, all fish were anaesthetised simultaneously at 0.15 ml/L incrementally at 0.05 ml/L for ease of handling and to avoid uncontrolled injuries from excitation (see Vaughan *et al.* 2008; Neiffer and Stamper 2009).

Each fish was assigned a random number between 1 and 126 and an individual sampling code. Sixty-three anaesthetised fish were randomly selected and given a standardised, superficial unilateral lesion on the body mid-region, randomised on the right or left side per individual, by removing scales with sterile forceps from an area of 0.15 cm² defined using a notched plastic template. The opposite uninjured side served as an internal control per individual fish. Sixty-three fish remained uninjured. Nine of the injured fish, and nine uninjured fish were selected randomly for immediate baseline data collection (designated day 0) to provide data for the injured and non-injured sides of the injured fish and both sides of uninjured fish at the beginning of the experiment. The remaining 54 injured fish were randomly assigned to the treatment group "injured fish cohabited with shrimp" (n = 27) or the first control group "injured fish without shrimp" (n = 27). The remaining 54 uninjured fish were assigned to the second and third control groups "uninjured fish cohabited with shrimp" (n = 27), and "uninjured fish without shrimp" (n = 27). All fish were given approximately 15 minutes to recover fully from the effects of anaesthesia on day 0, before I commenced experimentation.

During the experiment, all fish were housed individually in separate labelled 3 L polyethylene aquaria coupled to the marine recirculating life-support system. Each fish was removed with a soft aquarium hand net and introduced to their designated identical individual treatment or control tanks containing or excluding an individual *L. amboinensis* for 1 hour exposure at 11:00 daily, repeated for a maximum of seven days (days 0–6). Shrimp were not transferred to fish as this interferes with their ability to clean. I was limited to a cohort of 29 individual adult *L. amboinensis*, and therefore each shrimp was re-used once daily for the groups "injured fish cohabited with shrimp" and "uninjured fish cohabited with shrimp". However, to avoid any potential repetitive individual effect, each of the 29 shrimp was assigned a different individual fish, for each hour's 'cleaning' per day thus avoiding the same fish-shrimp combination for the duration of the experiment. For this study, cleaning by the cleaner shrimp was considered purposeful direct contact between fish and shrimps' chelae. Three different cleaning contact locations were identified and categorised, namely shrimp with the sides of the fish, oral or ventral contact (Fig. 3.1a-c). All separate cleaning bouts were recorded using Panasonic HC-V180 high definition video cameras on 16 GB SDX memory cards and there was no human observation or disturbance during the 1 hour period. Following each 1 hour exposure, fish were either returned to their individual holding tanks, or processed for photography and immediately thereafter humanely euthanased as outlined in the ethical note earlier.

3.3.4 Photography and sodium fluorescein staining of injured tissue

To record an epithelial pigmentation change of the controlled superficial injury, and to quantify the area of damaged tissue from both the controlled injury site (the area of interest) and any non-specific injury, nine fish from each of the treatment and three control groups were removed after the 1 hour exposure to shrimp or no shrimp on days 2, 4 and 6. All fish sampled were

placed into individually labelled containers of seawater and anaesthetised with 2-phenoxyethanol again. Once anaesthetised, each fish was placed individually onto a purpose-built glass photographic stage with integrated scale bar and photographed under 30 W LED-produced white light delivered through a diffused lens at 2200 lumen using a Nikon D7000 DSLR digital camera and tripod, in a darkroom to provide skin colour data (Fig. 3.2a). Colour controls were incorporated directly into the photographic stage and designed specifically for use as controls in the C.I.E. (Commission Internationale de l'Éclairage) *L*a*b** colour space analyses, using "*leap frog*" (green; Taubmans; T15167.6) and "*hot lips"* (red; Dulux P05H9), and "*blue*" (Dulux; 06231) and "*high alert yellow*" (British Paints; A1439) paint colour standards for negative and positive values of both the *a** and *b** channels, respectively. Each fish was then placed into a second container of seawater-anaesthetic-sodium fluorescein solution (0.15 ml/L 2-phenoxyethanol; 200 mg/L sodium fluorescein) for six minutes for the sodium fluorescein to bind with damaged tissue (see Noga and Udomkusonri 2002). Thereafter, each fish was individually transferred to a container of seawater anaesthetic solution (0.15 ml/L 2-phenoxyethanol) to remove excess sodium fluorescein before being returned to the photographic stage to be photographed under 25 W (GL-UVB22) long-wave ultraviolet irradiation using the same camera setup, but ISO 1600 exposure (Fig. 3.2b), to detect any epithelial damage (Fig. 3.2c).

3.3.5 Image analyses

All photographs were taken in a controlled environment to maximise repeatability and to avoid any influence on images taken initially in the RGB colour space before transformation to the C.I.E. *L*a*b** colour space (Lab Color – 24 bit) (Svensson and Sköld 2011). To obtain information on any potential changes in epithelial pigmentation of the wound over time, digital photographs taken under white light (Fig. 3.2a) were converted to C.I.E *L*a*b** colour space

using Corel PHOTO-PAINT X7 licenced to DBV. The three channels were then split using the functions: *Image-Split Channels To-Lab* to reveal their individual histograms. The *a** and *b** channels were used for analyses in exclusion of luminance (*L**) because they operate independently of light intensity (Svensson and Sköld 2011). The histogram colour space level (-127 to 128) for peaks representing the four colour controls (two per channel) for the *a** and *b** channels were recorded first for each photograph. These colour control data were necessary to verify that initial control of environmental conditions during photography were adequate, and were subjected to statistical analyses. Thereafter, the wounded area section of the photographs was cropped and these sections further processed using the *a** and *b** channels, with the number of pixels recorded per colour space level. These raw data were then transformed to a percentage to standardise for fish size and were exported manually to a spreadsheet (the software currently does not support an automated function) for further statistical analyses.

Digital photographs taken under long-wave ultraviolet irradiation (Fig. 3.2b) were processed using FIJI image analysis software (Schindelin *et al.* 2012). Each RGB image was first calibrated to the known scale (1 cm; not shown), then converted to *CIELAB* and the image separated into its three channels (Fig. 3.2c) using the menu functions *Image_Stacks,* and *Stacks_to_images*. Both the *a** and *b** channels were scrutinised for the detection of the sodium fluorescein signal. Although the signal was detectable in both channels due to the emission range of sodium fluorescein (500–600 nm, Berkow *et al*. 1991; Fig. 3.2c), because of the possible influence on its peak emission by seawater (see Doughty 2010), the *b** channel was selected for image analysis (Fig. 3.2c). Image segmentation was performed on the *b** channel images using thresholding. The white scale bar (not shown) reflected close to 0 (neutral) on the image histogram with an accuracy of 97.8% (calculated as a percentage of the channel range, where 0=100%) across all images taken under ultraviolet irradiation (2.72 ± 1.31 (0–6, $n = 126$;

left fish side), and 2.78 ± 1.45 (0–8, n = 126; right fish side)). Therefore the threshold reference value was considered unlabelled pixels representing the scale bar at 0, and all pixels labelled above 0 (+) in the yellow range reflected the sodium fluorescein fluorescence signal. *Dark background* was selected as the default, and background pixels were set to *NaN*, allowing a full threshold view of all labelled pixels in default red against a black background (Fig 3.2c). Each area of interest (AOI) was measured separately from any non-specific (Nonsp) areas of damaged tissue as the area (in cm^2) of labelled pixels calibrated to the scale bar. All data per image were exported to a spreadsheet for further statistical analyses.

3.3.6 Statistical analyses

Data for behaviour, white light photography (for analysing colour), and long-wave ultraviolet photography (for tissue damage) were analysed separately because each subset had different treatment levels (see below). All analyses were performed in R (Version 3.4.0; R Development Core Team 2017), using the packages 'lme4' (Bates *et al*. 2015) for mixed effects random-intercept models, 'stats' for linear regression models, and 'lmPerm' (Wheeler and Torchiano 2016) for permutation tests. Six missing values for the colour dataset were imputed using the package 'Hmisc' (Harrell *et al*. 2017) using the function 'areglmpute()' with 5 imputations. All data, except those for colour analyses were log-transformed before analysis, which successfully homogenised the variances and produced normally-distributed residuals; all separate models passed diagnostic scrutiny. For colour analyses the log-transformed data still did not satisfy test assumptions, and I therefore used permutation tests ('lmPerm'), which do not assume homogenous variances, or normally distributed residuals.

3.3.6.1 Analysis of behavioural data

I examined the effects of treatment (two levels: *Injured_with_shrimp*; *Uninjured_with_shrimp*), day, and cleaning contact location with respect to total cleaning time (the response variable) using a series of mixed effects random intercept models (see Appendix 2). To allow for variation between individual fish and shrimp, and to accommodate repeated measures, I treated the fish and the shrimp both as random effects in all these analyses.

The initial exploratory model investigated all cleaning contact locations (fish sides, oral, and ventral) recorded on the fish per day testing the response variable '*cleaning time*' as a function of the fixed effects '*day*', '*cleaning contact locations*', and interaction '*day* x *cleaning contact locations*', and '*fish*' and '*shrimp*' as random effects. Thereafter, I repeated this analysis using a subset excluding oral and ventral cleaning contact locations. This was done to examine whether cleaning behaviour differed between injured and uninjured sides of the fish. Because both injured and uninjured fish were included in the trial, the fish side had four levels (1. Injured side on injured fish; 2. Not injured side on injured fish; 3. Left side on uninjured fish; 4. Right side on uninjured fish) allowing the effects of injury on cleaning times to be compared both within and between fish.

With this dataset, two alternative approaches were used to model temporal change in cleaning times (the response variable). In the first, I treated day as a numeric variable, included in the model as either a quadratic or a linear function to test for curvature testing the response variable '*cleaning time*' as a function of the fixed effects '*cleaning contact locations*', '*day*', 'day^2', and the interactions '*cleaning contact locations with day*', '*cleaning contact locations with* day^2', and '*fish*' and '*shrimp*' as random effects. This analysis was carried out twice, once including all of the data, and once excluding day 0, to determine whether any temporal changes in behaviour extended beyond the initial establishment period of injury (see Appendix 2). In

the second approach, based on the results of the first, the data were re-analysed with day treated as a category having two binary classes, *'day0'* and *'>day0'*.

3.3.6.2 Analysis of jolting

Additional behavioural observations in both injured and uninjured fish included occasional client fish jolting (n = 132 separate observations) while being cleaned by the cleaner shrimp. These jolts were usually followed by aggression shown towards the shrimp, where the fish would 'mouth' the shrimp or push it away. The number of '*jolts*' (the response variable) were analysed using a random-intercept mixed effects model with '*fish*' and '*shrimp*' as random effects, and '*treatment*' (two levels: *Injured_with_shrimp*; *Uninjured_with_shrimp*), and '*day*' as fixed effects (see Appendix 2).

3.3.6.3 Colour analyses

As a measure of the effect of shrimp presence on the colour of the injury (the response variable) of individual injured fish, the red and yellow spectra (positive values for a^* and b^* channels, respectively, reflecting the skin pigments, and visible rubor, were analysed using linear models as the data did not include any repeated measures (see Appendix 2).

The initial analysis aimed to determine whether the immediate post-injury period (between day 0 and day 2) produced a change in either the red or the yellow spectra. It compared three independent groups of injured fish: 1. day 0 immediately post-injury, not exposed to shrimp; 2. day 2 with shrimp; 3. day 2 without shrimp. This analysis used a one-way analysis of variance, because there was only a single baseline group with which to compare the groups with and without shrimp on day 2.

Then a subsequent analysis examined spectral differences between groups with and without shrimp over the subsequent period to examine whether the presence of shrimp affected

the spectral properties of the injury site as the injury healed. For this analysis, '*day*' (either 4 or 6), '*shrimp presence*' and the '*shrimp* x *day*' interaction were all included as potential explanatory variables.

3.3.6.4 Tissue damage analysis

The controlled injury I stained with sodium fluorescein to quantify the area of controlled damage with image analysis, fluoresced well immediately after the injury had been given (day 0), reflecting an average area of damage of 0.15 $cm^2 \pm 0.07$ ($n = 63$), but the same area of injury was not detectable with staining on days 2, 4, and 6 post-injury, suggesting that no additional fresh assault had taken place post-injury. Therefore only the non-specific damage was included in the statistical analysis. Non-specific tissue damage area (the response variable) calculated from the sodium fluorescein fluorescence using FIJI image analysis was investigated between two levels of four treatment groups (*With shrimp* and *Without shrimp; Injured fish* and *Uninjured fish*) over time to determine their effects on tissue damage. I used mixed effects random-intercept models with the '*fish*' as the random effect to allow for photographic sampling on both sides of the same fish. The response variable '*tissue damage area*' was tested as a function of the fixed effects '*treatment*', '*side*', '*day*' (as a factor). Two levels of fish sides were included in the analyses representing injured and uninjured sides (see Appendix 2).

3.4 Results

3.4.1 Analysis of behavioural data

The cleaner shrimp *L. amboinensis* interacted with injured and uninjured fish a total of 3,131 and 3,560 times, respectively, representing approximately 8% of the total observation time. To solicit cleaning, the client fish postured to the cleaner shrimp submissively by flaring open their opercula, opening their mouths, or by stretching out their fin rays. Client fish regulated shrimp

'cleaning', presenting specific sides of their body to the shrimp they wanted cleaned, or providing access to the inside of their mouth or their ventral area (Fig. 3.1a-c). Fifty four fish were used in these analyses. There were clear differences in cleaning times at different cleaning contact locations [Wald test: $X^2_{(5, n = 54)} = 377.92$, $p < 0.001$]. Fish predominantly presented their sides to shrimp for cleaning (Fig. 3.1a) over oral or ventral cleaning contact locations. Each cleaning bout lasted an average of 12.05 seconds ± 0.15 SE with an average of approximately 22.7 side, 1.5 oral, and 0.7 ventral cleaning bouts per fish per hour.

Shrimp spent less time cleaning injured fish on day 0 following infliction of the wound (Fig. 3.3). On day 0, cleaning times were significantly lower on the injured side of injured fish than they were on the uninjured side of injured fish, and either side of uninjured fish [Wald test: $X^2_{(3, n = 54)} = 9.32$, $p = 0.025$; Table 3.1]. However, after day 0, these injured fish allowed shrimp to clean for longer periods of time on both sides, and there were no further significant effects of the injury treatment, either when comparing injured sides with uninjured sides of injured fish, or when compared with uninjured fish. In particular, when day 0 was excluded from analyses, there was no significant effect of the cleaning contact locations, treatment, or day on cleaning times [Wald tests: $X^2_{(3, n = 54)} = 2.74$, $p = 0.43$; $X^2_{(1, n = 54)} = 0.01$, $p = 0.91$; $X^2_{(1, n = 54)} = 0.02$, $p = 0.88$, respectively].

3.4.2 Analysis of jolting

Fifty four fish were used in this analysis. There were no significant effects of either treatment or day on cheating [Wald tests: $X^2_{(6, n = 54)} = 4.39$, $p = 0.62$; $X^2_{(1, n = 54)} = 1.43$, $p = 0.23$, respectively]. However, the fish contributed approximately 20 times more variance in the cheating analysis model than shrimp. This most notably resulted from the same 10 individual fish repeatedly demonstrating jolting and aggressive behaviour towards different shrimp (Fig. 3.4).

3.4.3 Colour analyses

Sixty three fish were used in these analyses. No significant changes from baseline values or differences between groups with and without shrimp were observed at the injury site in the yellow spectrum [ANOVA: $F_{2,24} = 2.42$, $p = 0.10$; $F_{2,33} = 0.60$, $p = 0.55$].

The red spectrum, however, did show significant changes. Redness increased in the immediate post-injury period between day 0 and day 2 [Fig. 5; ANOVA: $F_{2,24} = 3.41$, $p = 0.049$] , with little evident difference in the size of the increase for groups with and without shrimp (Fig. 3.5). In days 4 and 6, however, average injury redness was significantly lower in fish with shrimp than in fish without shrimp [Fig. 5, ANOVA: $F_{2,33} = 4.28$, $p = 0.02$]. In the groups without shrimp, injury redness by day 6 remained very close to the peak levels reached on day 2 (Fig. 3.5).

To confirm the accuracy of the photographic data used in these models, the colour controls were analysed using an independent samples *t*-test, revealing no significant difference between photographs [*a** channel red, *t* test: $t_{68.5} = 1.21$, $p = 0.22$; *b**; channel yellow, *t* test: $t_{69.3} = 0.24$, $p = 0.80$].

3.4.4 Tissue damage analysis

The baseline non-specific damage (day 0; Fig. 3.6) was excluded from the statistical analyses because of the confounding effect of creating the controlled injury on day 0. Therefore, 108 fish were used for these analyses. There was no significant difference in the non-specific damage (Fig. 3.7) between fish sides [Wald test: $X^2_{(1,\, n = 108)} = 0.97$, $p = 0.32$], or between injured and uninjured fish [Wald test: $X^2_{(1,\, n = 108)} = 3.50$, $p = 0.06$]. Therefore, shrimp were not the cause of the non-specific skin damage. The non-specific damage was however significantly less in fish cohabited with shrimp [Wald test: $X^2_{(1,\, n = 108)} = 4.14$, $p = 0.04$], but also significantly less on day 4 than on either days 2 or 6 [Wald test: $X^2_{(2,\, n = 108)} = 12.7$, $p = 0.001$].

3.5 Discussion

This study presents the first investigation to empirically demonstrate that the cleaner shrimp *Lysmata amboinensis* attend to injured fish, and that visits to shrimp by injured fish have health benefits for these client fish. Observations of injured reef fishes in the Caribbean by Foster (1981) suggested that fishes with new injuries spend more time being cleaned by cleaner fishes on the reef than those in an advanced state of healing. However, Foster (1981) did not observe when these injuries were sustained. In our experimental trial, injured fish regulated the cleaning bouts with cleaner shrimp by presenting specific sides of their body to the shrimp to be cleaned more frequently. Immediately post-injury (i.e. day 0), fish reduced cleaning to the injured side of their body. This behavioural response by the fish may be specific to being cleaned by cleaner shrimp, and may correspond with the wound healing process.

Healing of epithelial injuries in fishes has been documented extensively (e.g. Fontenot and Neiffer 2004; Böckelmann *et al*. 2010). Post-injury, the stages of wound healing in fishes can be categorised by inflammation, re-epithelialisation, proliferation, organisation, and differentiation (Fontenot and Neiffer 2004). An inflammatory response in fishes may be evident from 1 hour post-injury, while concurrently, epithelial cells begin migrating across the injury boundary (Fontenot and Neiffer 2004). Re-epithelialisation of the injury by a single-cell layer of undifferentiated epithelial cells occurs rapidly to seal off the injury, and is completed in most fishes within 12 to 24 hours, followed by the subsequent stages of healing between 9 and 48 hours thereafter (see Fontenot and Neiffer 2004). This may help to explain the apparent reluctance of injured fish to present their injured side for cleaning post-injury in our trial. After 24 hours, all fish, regardless of treatment, showed no preference for cleaning on either side, which is suggestive of re-epithelialisation of the injury.

There was lack of detectable skin damage in the induced injury (area of interest) using sodium fluorescein on subsequent sampling days 2, 4 and 6 post-injury. This is supportive of

the rapid sealing of the injury by a single-cell layer of undifferentiated epithelium, as sodium fluorescein only binds to damaged cells (see Noga and Udomkusonri 2002). However, this method detected non-specific epithelial damage across all treatment groups for the duration of the experiment. This non-specific damage can be attributed to handling of individual fish, as much of this superficial damage was associated with the fin margins or the mouth area due to netting (Fig. 3.7; *cf*. Marcusso *et al.* 2014), and would have been fresh, non-accumulated damage. This is also supported by the analyses, where there was no significant difference in non-specific skin damage values between fish sides or between injured and uninjured fish, even though day 4 recorded significantly less non-specific damage across the experiment than days 2 and 6. Consequently, this evidence also demonstrated that the shrimp neither aggravated the existing controlled injury, nor created additional non-specific damage. On the contrary, their presence had a significant reduction effect on non-specific injury.

Cheating by cleaner organisms, including shrimp, is extensively documented in the literature (see review by Vaughan *et al.* 2016; Chapter 2) as the removal and consumption of client mucus, skin and scales by the cleaner. The long-wave ultraviolet analyses assumed that cheating by cleaner shrimp would be presented by exploitation of existing injuries by the shrimp, and thus the analyses were highly sensitive to epithelial damage. Arguably however, these analyses could not rule out potential cheating by mucus removal without disturbing the underlying epithelium. If cleaner shrimp did cheat, the question remains why they would avoid exploiting an existing injury. Certainly, other organisms do exploit injuries in other cleaning interactions (see Weeks 2000). It is this dilemma that, based on our analysis, suggests that the cleaner shrimp may not have cheated.

Currently, cheating by cleaner shrimp is assumed by proxy (Chapuis and Bshary 2009). Client fish jolt rate is considered a measure of cheating by cleaner fishes (Bshary and Grutter 2002; Whiteman and Côté 2002; Soares *et al.* 2008b; Oates *et al.* 2010). In contrast, little

comparative information exists for cleaner shrimp, although Chapuis and Bshary (2009) provided evidence that the long-arm cleaner shrimp, *Ancylomenes longicarpus* induced a similar client response (jolts) to cheating cleaner wrasse, *Labroides dimidiatus*, and Titus *et al.* (2017) recently used client jolts as a proxy for cheating by *Periclimenes yucatanicus*. Several client fish jolts were observed in the video footage taken during our cleaning experimental trial, often followed by reciprocal retaliatory client responses, considered in the literature as 'cleaner punishment' (Bshary and Grutter 2002, 2005). However, our analysis did not support the consideration that jolt rate is a good proxy for cheating by cleaner shrimp. Each fish was cohabited with a different shrimp daily, and the same fish-shrimp combination was never repeated, yet, some of the same individual fish displayed repeated jolting and aggression towards the different shrimp over different days. Of these 54 fish, including both injured and uninjured fish, a total of only 19 (35%) recorded any jolting behaviour, i.e. 65% of fish did not jolt at all. Of these 19 fish however, 10 individuals displayed repeated jolting behaviour and aggression towards shrimp (see Fig. 3.4). One possible explanation is that individual fish have different tolerance levels to discomfort imposed by shrimp during cleaning interactions, and therefore some fish may repeatedly react more frequently by jolting than others to the same stimuli, whether these be directly related to cheating, or not.

The concept of cheating in cleaning symbiosis implies the removal of a food resource by the cleaner, which carries an associated temporary cost to the client (Gorlick 1980). It is therefore assumed that the removal and consumption of mucus for example, which is costly in energy to produce, has a negative impact on the client. However, this actual cost of mucus removal by cleaners is unknown (Eckes *et al.* 2015), and is likely unquantifiable. The constituents of fish mucus change with the health status of the fish, and the influence of stress, pathogen invasion, and changes in environmental conditions (Shephard 1994). One of the most significant attributes of mucus is the role of defence against pathogen invasion, and as such,

represents a renewable surface, which functions to remove potential pathogens (Shephard 1994). Cleaner shrimp, by removing and consuming fish mucus, may actually be promoting the renewal of the mucus layer, and in so doing removing older surface layers of mucus along with entrapped potential pathogens. This would ultimately provide an increased benefit to the client, especially one which presented with an injury, and might support recovery.

My data suggest that the cleaner shrimp had an indirect positive effect on wound healing which may support this hypothesis. After an initial increase, injury redness decreased in fish cohabited with the cleaner shrimp, but remained consistently high in fish without cleaner shrimp (see Fig. 3.5). This suggests that initial redness is related to the reaction of the tissue to injury, but that the cleaning by these shrimp influenced the injury at the tissue-level. These shrimp did not appear to focus cleaning specifically around the injury site, but their general cleaning activity did influence healing, demonstrated by both the reduction of rubor, and the reduction of non-specific epithelial damage in fish cleaned by shrimp. As in other vertebrates, rubor (or redness) is part of the acute inflammatory response in fish, and is a consequence of increased blood flow to the area (hyperaemia) and/or haemorrhage (Roberts 2012). Sites of injury in fish are vulnerable to colonisation by opportunistic pathogens (Fontenot *et al.* 2004; Jensen *et al*. 2015) largely due to breaching of the host's physical and humoral defence mechanisms, but possibly also due to the presence of increased levels of blood products. Cleaner shrimp are also known to indirectly influence the health of client fishes by reducing cortisol levels as a function of cleaning (Bshary *et al.* 2007). This phenomenon has also been documented for cleaner fish (Soares *et al*. 2011). Cortisol is an important corticosteroid in fishes, and is known to reduce mitogenesis, and therefore impairs healing, but it also reduces antibody production, and general resistance to pathogens (Castro *et al.* 2011). Therefore, cleaning by shrimp may act synergistically by reducing the successful invasion of injuries by

pathogens through their own feeding behaviour but also by supporting the client's own defence and stress-reduction mechanisms.

Acknowledgements

I am grateful to Cairns Marine, Cairns, Australia, and in particular to Laura Simmons for the ongoing support for the investigations into cleaning symbiosis. I acknowledge the generous assistance of volunteers Alejandro Trujillo González, Thienthanh Trinh, Julian Wilson, and Pauline Narvaez. This chapter is dedicated *in memoriam* to Patrick Donnelly.

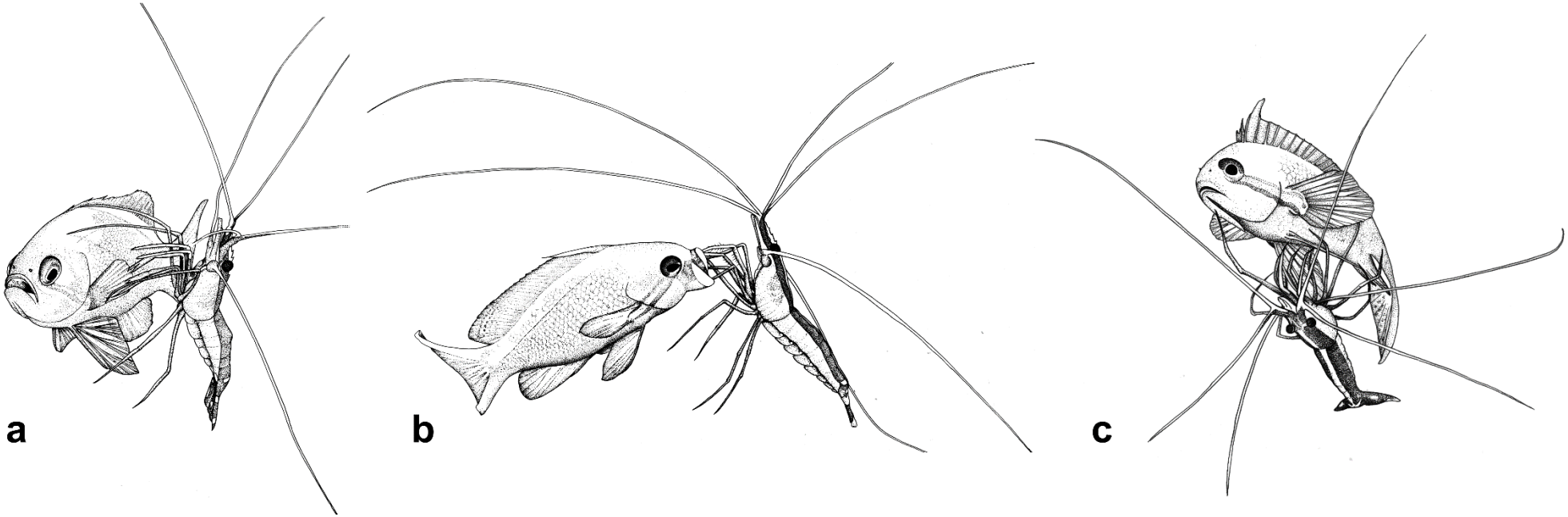

Fig. 3.1. Different cleaning contact locations between *Lysmata amboinensis* and *Pseudanthias squamipinnis* from which data were generated: **a.** sides of fish; **b**. oral; **c.** ventral.

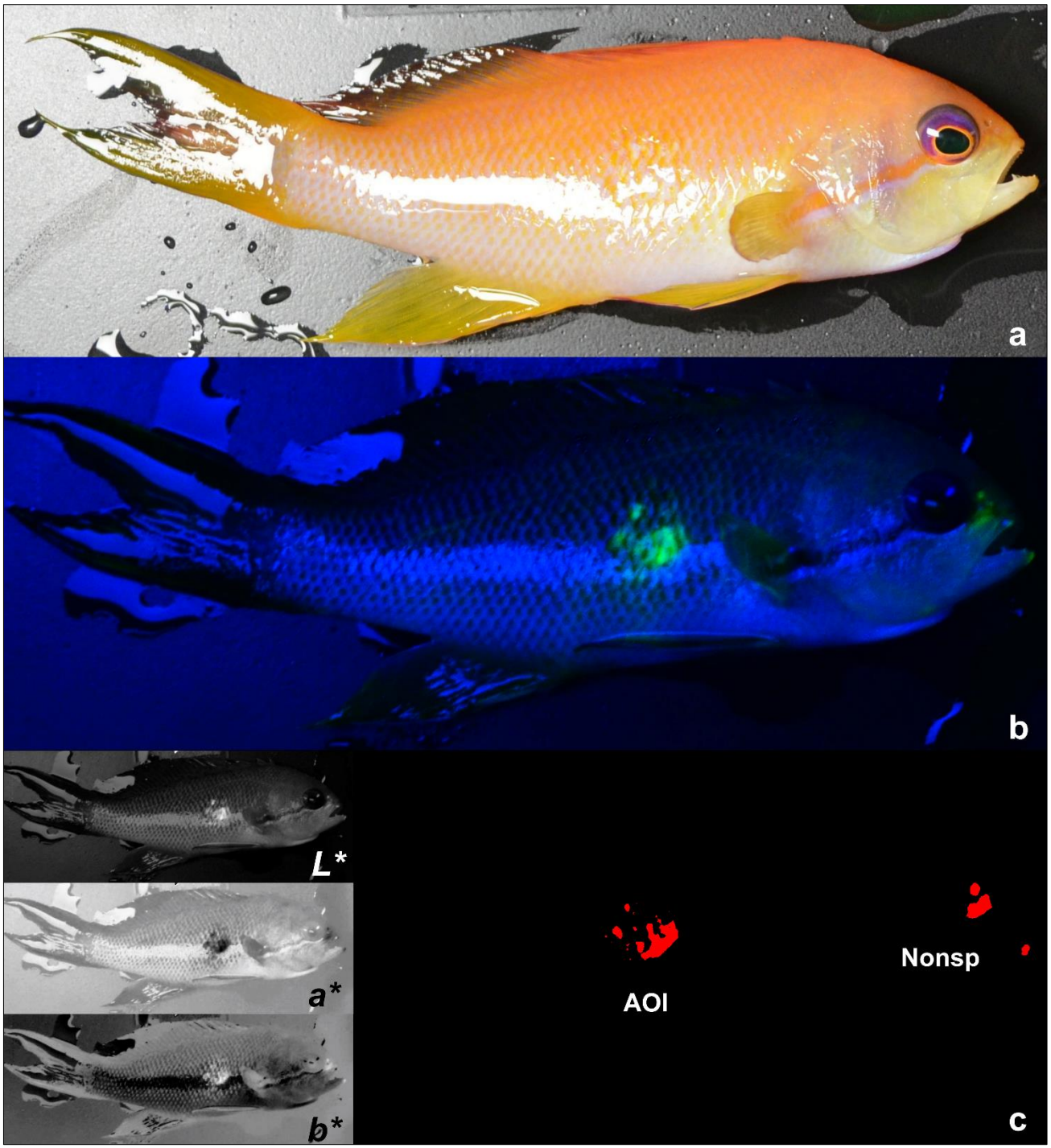

Fig. 3.2. Photography and image analysis examples of injured fish demonstrating the unstained injury under white light (**a**), the stained injury using sodium fluorescein under long-wave ultraviolet (**b**), and the threshold output image from image analysis (**c**) produced from the C.I.E. (Commission Internationale de l'Éclairage) *L*a*b** colour space-converted original RBG (Red, Blue, Green) long-wave ultraviolet image, using channel *b** from the available channels *L** (luminance), *a** (red and green spectra), *b** (yellow and blue spectra). Abbreviations: AOI = area of interest (the controlled deliberate unilateral injury); Nonsp = non-specific area of epithelial damage.

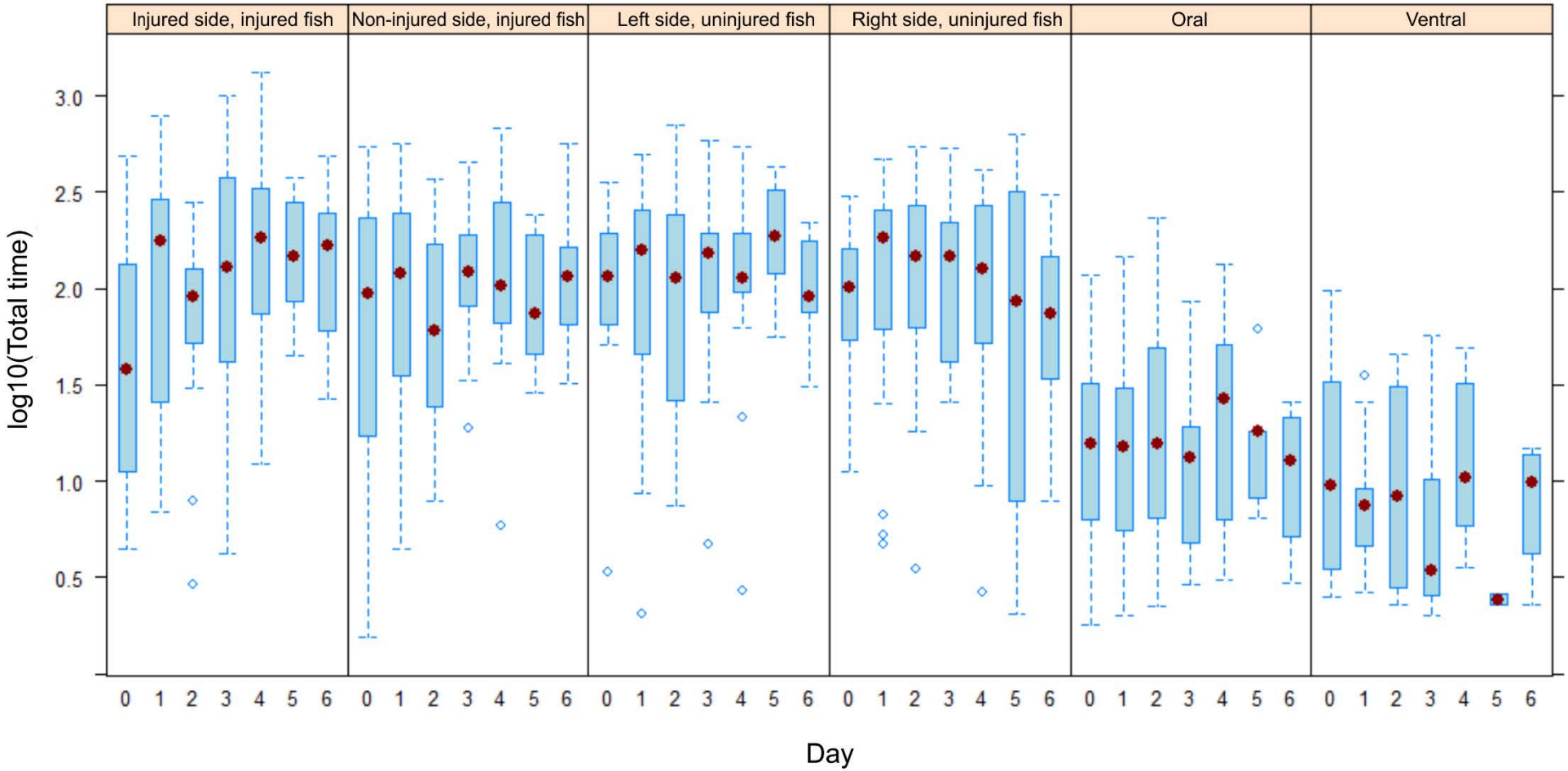

Fig. 3.3. Total cleaning time (log-transformed) per day for the different cleaning contact locations between *Lysmata amboinensis* and *Pseudanthias squamipinnis* demonstrating an initial reluctance of injured fish to have their injured side cleaned by shrimp on day 0. Nine fish per observation.

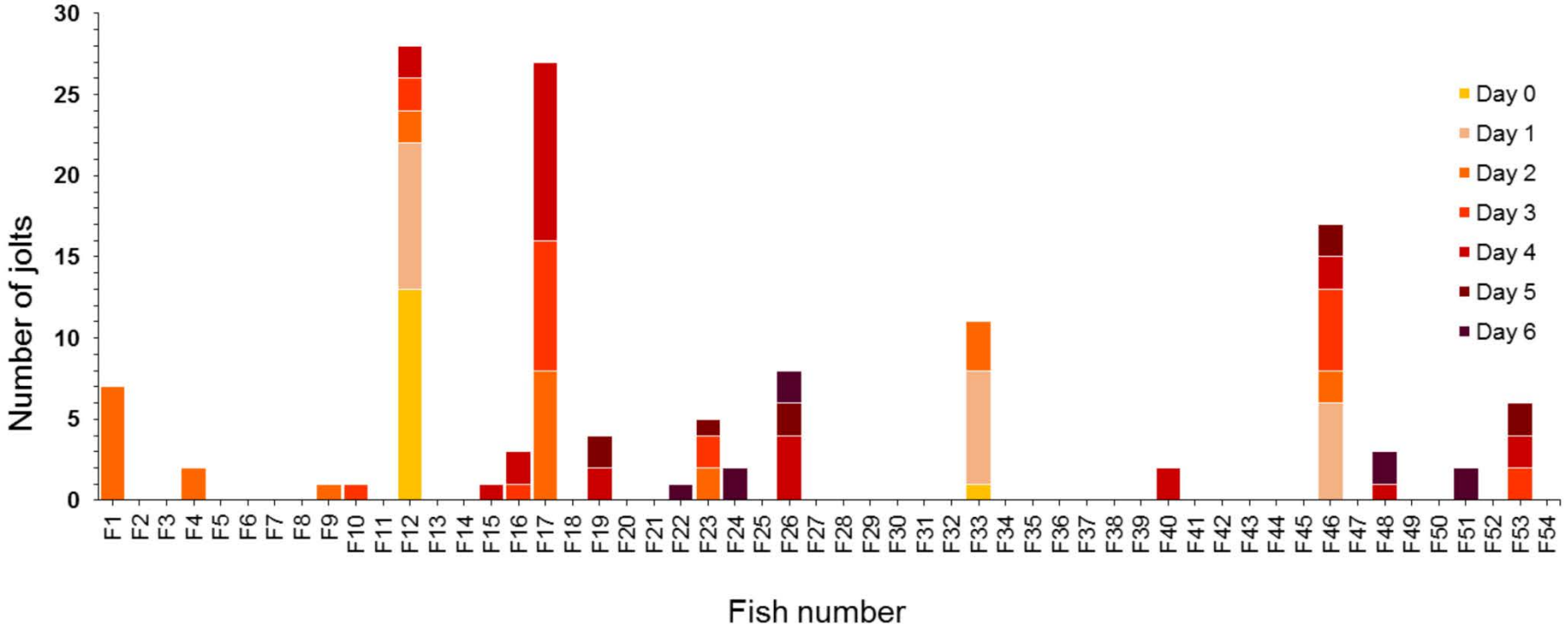

Fig. 3.4. The cumulative number of jolts recorded per individual fish over 7 days' exposure to different cleaner shrimp daily for 1 hour, demonstrating the idiosyncrasy of individual fish, and how this may influence the consideration of jolt rate as a proxy for cheating in a cleaning symbiosis.

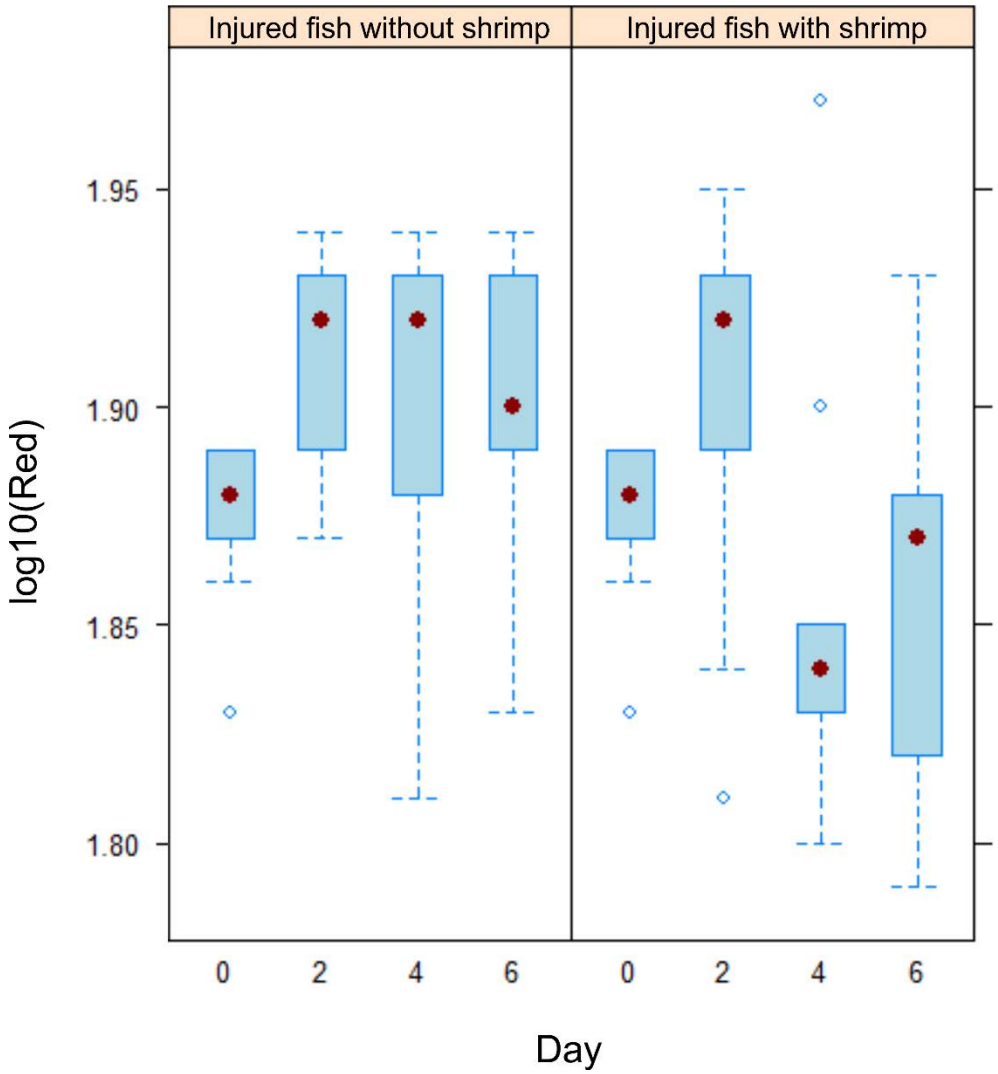

Fig. 3.5. The reduction effect of *Lysmata amboinensis* on the redness of the controlled injury of injured fish in colour photographs after initial injury induction on day 0 (the baseline). Nine fish per observation.

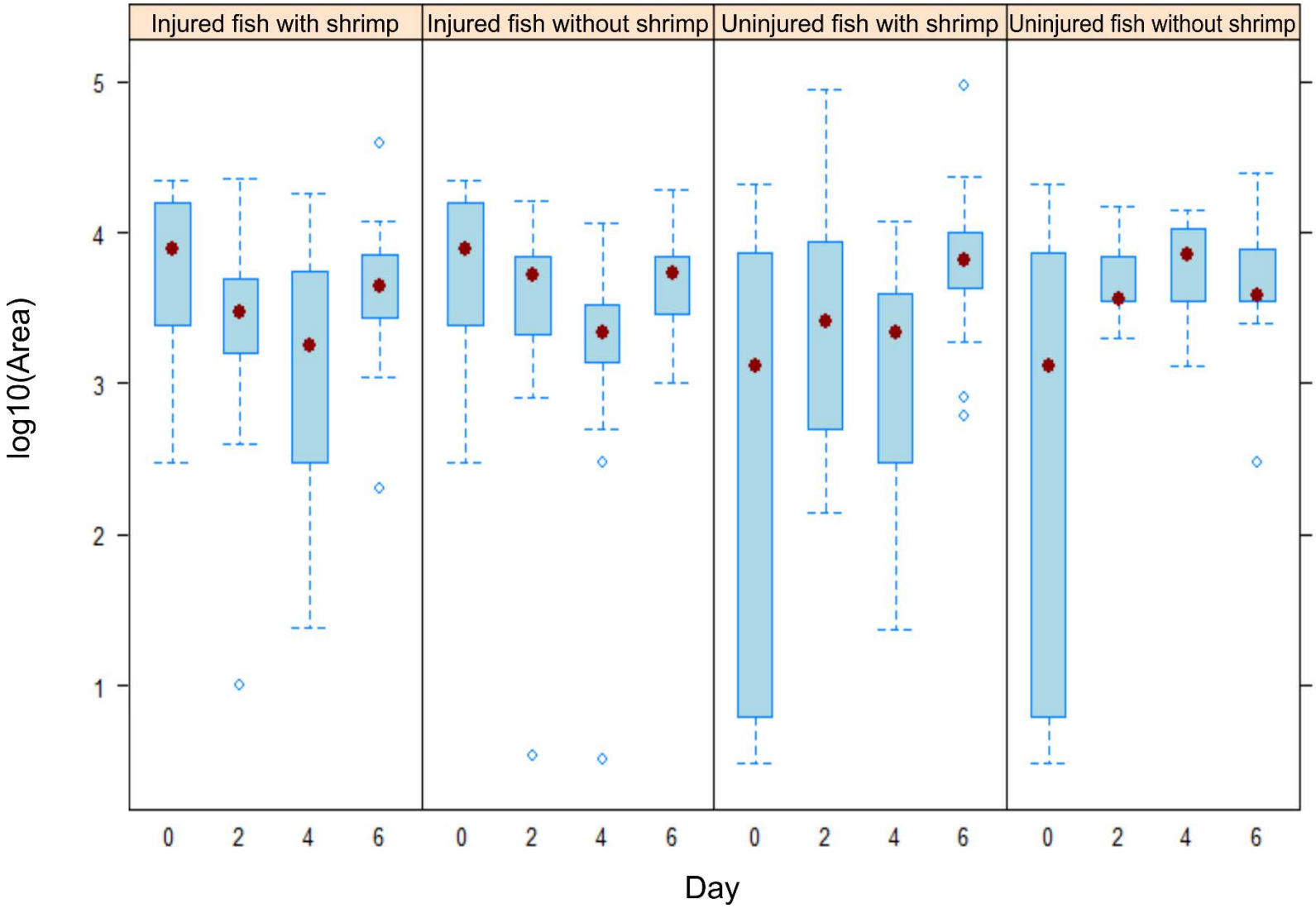

Fig. 3.6. Non-specific skin damage of injured and uninjured fish, with and without shrimp after day 0 (the baseline), demonstrating that non-specific skin damage did not resulted from shrimp, but rather daily fish handling. Nine fish per observation.

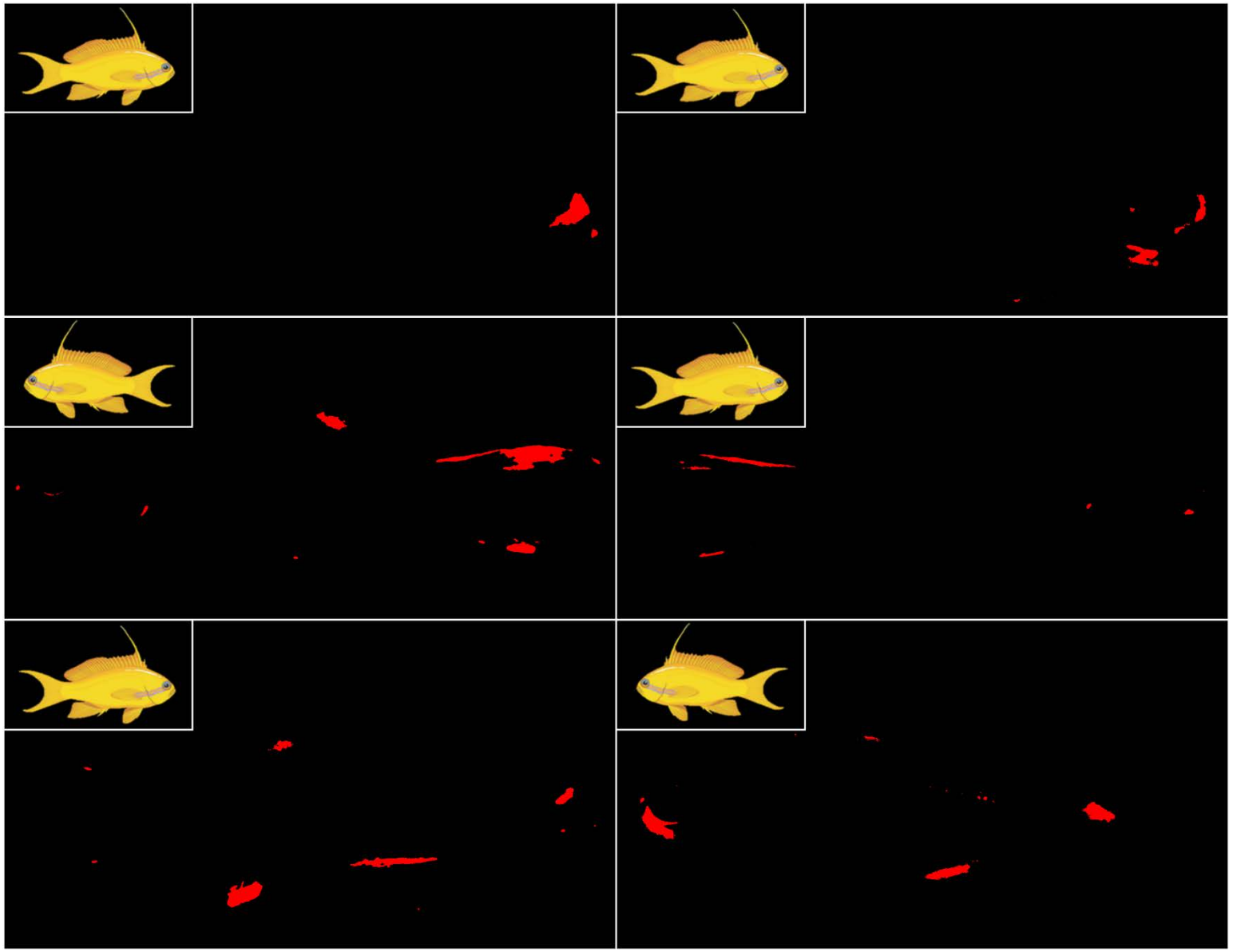

Fig. 3.7. Examples of non-specific skin damage on *Pseudanthias squamipinnis* associated with handling.

Table 3.1. Confidence intervals for the fixed effects in the cleaning time mixed effects model.

Fixed effects	**2.5%**	**97.5%**
(Intercept)	1.854	2.168
Uninjured side of injured fish	-0.231	0.036
Left side of uninjured fish	-0.210	0.189
Right side of uninjured fish	-0.257	0.142
isDay0TRUE	-0.717	-0.233
Uninjured side of injured fish: isDay0TRUE	-0.059	0.609
Left side of uninjured fish: isDay0TRUE	0.137	0.820
Right side of uninjured fish: isDay0TRUE	0.112	0.770

CHAPTER 4

Cleaner shrimp are a sustainable option to treat parasitic disease in farmed fish

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In this chapter, I test the efficacy of four different cleaner shrimp species against three economically important ectoparasites of culture fish to address the third thesis aim.

4.1 Abstract

Chemical use is widespread in aquaculture to treat parasitic diseases in farmed fish. Cleaner fish biocontrols are increasingly used in fish farming as an alternative to medicines. However, cleaner fish are susceptible to some of their clients' parasites and their supply is largely dependent on wild harvest. In comparison, cleaner shrimp are not susceptible to fish ectoparasites and they can be reliably bred in captivity. The effectiveness of shrimp in reducing parasites on farmed fish remained unexplored until now. I tested whether four cleaner shrimp species reduced three harmful parasites (a monogenean fluke, a ciliate protozoan, and a leech) on farmed grouper. All shrimp reduced parasites on fish and most reduced the free-living early-life environmental stages of the parasites – a function not provided by cleaner fish. Cleaner

shrimp are sustainable biocontrol candidates against ectoparasites of farmed fish, with the peppermint cleaner shrimp (*Lysmata vittata*) reducing parasites by up to 98%.

4.2 Introduction

Consumers are becoming increasingly aware of the impacts that their food choices have on the environment. In response, products that are produced sustainably communicate this sustainability through eco-labelling, which in turn informs client choice (Grunert 2011). Farmed fish products are perceived as likely to contain higher amounts of antibiotics or other chemicals, and to be generally less healthy than wild-caught fish (Banarjee *et al.* 2014; Claret *et al.* 2014). Thus, more naturally-produced farmed products are required.

Chemical therapy against parasitic disease remains commonplace in global aquaculture. There is considerable overlap in the antibiotics used in human medicines and animal food production, notably in aquaculture (Done *et al.* 2015; Watts *et al*. 2017), but other chemicals, such as organophosphates, avermectins, pyrethroids, and benzoylureas are also used (Aaen *et al.* 2015) with origins in agricultural pest control. Some of these chemicals may have negative environmental impacts (Langford *et al.* 2014), but concerns have also been raised of resistance of fish parasites to some of these chemicals when used in aquaculture (Costello *et al.* 2001; Costello 2006; Lees *et al*. 2008; Jones *et al*. 2013; Langford *et al.* 2014; Aaen *et al.* 2015).

Parasitic diseases (i.e., excluding viral and bacterial aetiologies) account for between 30% and 50% of annual stock losses in certain aquaculture industries in Asia (Shinn *et al.* 2015). Asia is the world's largest aquaculture producing region (FAO 2016; Subasinghe 2017) of which Asian seabass (barramundi, *Lates calcarifer* (Bloch, 1790)) and groupers (*Epinephelus* Bloch, 1793 spp.) contribute a significant proportion to the marine finfish sector (Asia-Pacific Fisheries Commission 2014; Shinn *et al*. 2015). In general, diseases influencing aquaculture are broadly categorised into three main groups. These include 1) diseases that potentially affect trade and are listed by the World Organisation for Animal Health (OIE), 2)

diseases that affect cultured species at various production levels, and 3) emergent diseases (Subasinghe 2017). In Asian fish aquaculture, diseases arguably represent the latter two groups.

Of the many parasitic species recorded for marine finfish species in the Asia-Pacific region, the ciliate *Cryptocaryon irritans* Brown, 1951, the monogenean *Neobenedenia girellae* (Hargis, 1955), and the leech *Zeylanicobdella arugamensis* de Silva, 1963 have economic significance, and are directly responsible for financial losses due to mortalities, or poor production performance (Shinn *et al*. 2015). Additionally, these parasites share similar life history strategies; each has a direct life-cycle, and a diverse fish host range. The environmental stages (tomont cysts, eggs, or cocoons, respectively) are resistant to chemical therapies, resulting in eradication difficulties, imminent reinfections post fish treatment, and ongoing chronic-level disease. These characteristics, coupled with a broad tropical and sub-tropical geographic range (de Silva 1963; Ogawa *et al.* 1995; Cruz-Lacierda *et al.* 2000; Mo *et al.* 2016), contribute to their success in captive fish populations in this region, and elsewhere.

Employing biocontrols against parasites in aquaculture may reduce chemical intervention use. The salmon, *Salmo salar* Linnaeus, 1758 aquaculture industry is the largest producer of cultured finfish in Europe (Clarke and Bostock 2017), and is currently the only aquaculture industry globally that employs native cleaner fish as biocontrols to effectively control parasites (Liu and vanhauwer Bjelland 2014; González and de Boer 2017; Powell *et al*. 2017). Currently, no equivalent cleaner biocontrols are used commercially in sub-tropical or tropical aquaculture. Unfortunately, sub-tropical or tropical cleaner fishes would likely be susceptible to some of the parasites they would be employed to control, especially those with a low host-specificity e.g. *C. irritans* (see Burgess and Matthews 1995), and some monogeneans (Whittington and Deveney 2011). Therefore, it is unlikely that a teleost cleaner biocontrol would be feasible for sub-tropical or tropical marine aquaculture. In contrast, cleaner shrimp, which are active predators of fish ectoparasites (Bunkley-Williams and Williams 1998;

Becker and Grutter 2005; Östlund-Nilsson *et al.* 2005; McCammon *et al*. 2010; Militz and Hutson 2015; Chapter 2), are not susceptible to these parasites of fishes. However, they have never been directly evaluated as biocontrols in aquaculture.

Here, I investigated the ability of four cleaner shrimp species, *Lysmata amboinensis* (de Man, 1888), *Lysmata vittata* (Stimpson, 1860), *Stenopus hispidus* (Olivier, 1811), and *Urocaridella antonbruunii* (Bruce, 1967) to reduce the parasites *C. irritans*, *N. girellae*, and/or *Z. arugamensis* (representing a protozoan, a monogenean, and a hirudinean fish ectoparasite) infesting the susceptible aquaculture host fish *Epinephelus coioides* (Hamilton, 1822) (orange-spotted grouper), and the parasites' respective tomont (cyst), egg, and cocoon environmental stage, under controlled laboratory conditions. I evaluated the ability of shrimp to perform diurnally and/or nocturnally, and provide for the first time an insight into different shrimp species' preferences and abilities. The results highlight and support the further investigation of *Lysmata vittata* as the first cleaner shrimp biocontrol candidate for sub-tropical and tropical aquaculture.

4.3 Methods

4.3.1 Animal ethics

Ethics approval was granted under the James Cook University Ethics Committee Permit numbers A2260, and A2457, conforming strictly to the national regulations set out in the National Health and Medical Research Council (2013) Australian code for the care and use of animals for scientific purposes, 8th edition, under Section 39 of the National Health and Medical Research Council Act, 1992. *Neobenedenia girellae* and *Z. arugamensis* could be recovered from fish without destructive sampling. Therefore, all fish used in these challenge experiments were returned to their original holding area after experimentation. *Cryptocaryon irritans* is a subcutaneous fish parasite but also infests the gill tissue. For accurate counts on

fish it was therefore necessary to humanely euthanase all fish in the *C. irritans* challenges using an anaesthetic overdose of 2-phenoxyethanol at 1.5 ml/L for 10 minutes (Neiffer and Stamper 2009) prior to sampling.

4.3.2 Animal acquisition, maintenance and biosecurity

Thirty individuals each of the cleaner shrimp *L. amboinensis*, *L. vittata*, *S. hispidus*, were purchased from a commercial, Australian ornamental fish/invertebrate supplier (Cairns Marine) and 30 *U. antonbruunii* individuals were collected using SCUBA off Lizard Island, Queensland, Australia. All fish used *in vivo* in the experiment (*E. coioides*, *Epinephelus lanceolatus* (Bloch, 1790), *L. calcarifer*), were obtained from commercial hatcheries in Queensland, Australia. All fish were given a 5-minute freshwater bath on arrival in the laboratory to remove potential ectoparasites. The fish were then quarantined in five 70 L plastic aquaria coupled to a recirculating marine life-support system for 30 days before being transferred to a single 5000 L tank prior to the experiment. All cleaner shrimp were housed separately in individual 3 L plastic aquaria connected to a separate recirculating marine life-support system. Prior to experimentation, fish were fed daily with 3 mm juvenile marine fish floating pellets (Ridley Agriproducts Pty Ltd; product code: 107578) and shrimp were given defrosted *Mysis* sp. shrimp daily, to satiation. During the experiments, shrimp were fed after removal from experimental tanks. To avoid the potential for cross-contamination, and to allow for effective biosecurity, each parasite-fish challenge experiment was run separately for each shrimp species, and equipment disinfected with 50 mg/L sodium hypochlorite between trials following OIE guidelines. All effluent water from experimentation was first disinfected with sodium hypochlorite at the same final concentration prior to disposal via sewer system.

4.3.3 Parasite source and monoculture methods

All parasites were obtained from species monocultures established in the Marine Parasitology Laboratory at James Cook University, Townsville, Australia. *Cryptocaryon irritans* was cultured continuously in the laboratory at 26°C and 35 ppt on individual juvenile *L. calcarifer* (~15 cm total length). Freshwater juvenile *L. calcarifer* were acclimatised to seawater over two days before being introduced directly into the parasite culture tank (60 L). These fish were then allowed to support a low intensity, single life-cycle of *C. irritans*. Once visible trophonts had vacated the host, the fish were removed from the culture, and re-acclimatised to freshwater (which kills any remaining parasites) before being returned to their freshwater aquarium. To boost numbers of *C. irritans* prior to experimentation, additional fish were added to the culture to increase tomont production for harvesting from the culture tank. Individual *L. calcarifer* were also used to maintain a continuous monoculture of *N. girellae* in the laboratory, following previously published methodology (Hutson *et al*. 2018). *Zeylanicobdella arugamensis* was cultured continuously at 30°C and 28 ppt on a cohort of 40 individual *E. lanceolatus* with an approximate average individual mass of 1.5 kg each (i.e. one life-cycle completion per individual, rotated through the culture). Adult leeches vacate the host to produce cocoons which they deposited directly onto the glass surfaces of the culture tank. Once adult leeches had begun producing cocoons, the existing host was removed from the culture, and replaced with a new host fish coinciding with the emergence of juvenile leeches.

4.3.4 Experimental design

Diurnal and nocturnal challenges were performed for the parasitic and the environmental stages of *C. irritans* and *N. girellae* for all shrimp species. The numbers of leeches were limited so I completed diurnal and nocturnal challenges for *L. amboinensis*, and diurnal challenges for *L.*

vittata and *S. hispidus* only (i.e. excluding *U. antonbruunii*). The ability of only *L. vittata* to reduce leech cocoons was evaluated.

A total of four hundred individual juvenile *E. coioides* (~15 cm in total length) were used for the parasitic stage challenges with *N. girellae*, *C. irritans*, and *Z. arugamensis* (i.e. 10 individual treatment and 10 individual control fish per challenge, for each parasite species), per shrimp species used (Fig. 4.1). The shrimp and client fish species were selected because they share an overlapping distribution in Australia and through parts of the Asia-Pacific, and the client is a valued aquaculture species in this region. For each parasitic stage challenge, infected fish were randomly assigned to 20 identical 3 L plastic aquaria (10 treatment and 10 control aquaria), coupled to a recirculating marine life-support system to maintain water quality conditions over the experimental period (24° C and 35 ppt; Fig. 4.1). Random numbers for fish assignment were generated using a random number generator.

For each parasitic stage challenge, 10 individual shrimp were cohabited with the 10 treatment fish only, and the experiment ran for 12 hours (either 12 hours diurnal; 07:00-19:00, or 12 hours nocturnal; 19:00-07:00, in an artificially light-controlled laboratory). All tanks were fitted with a tightly fitting 60 µm mesh cover secured over the water surface area by the fitted lid of each tank to allow water exchange without any potential parasite loss (Fig. 4.1).

After 12 hours, all fish were removed from their treatment and control tanks. Fish infected with *N. girellae* were placed into individual glass beakers containing dechlorinated freshwater and an anaesthetic concentration of 2-phenoxyethanol at 0.15 ml/L (Vaughan *et al.* 2008; Penning *et al.* 2017) for 5 minutes to anaesthetise the fish, and to kill and to dislodge *N. girellae*. Similarly, fish with leeches were placed into individual glass beakers, but containing a seawater-anaesthetic concentration of 2-phenoxyethanol to immobilise the fish for inspection. These fish were moved individually to a large glass Petri dish and observed at 20X magnification under a Leica M60 dissection microscope. Fish were kept submerged in the same

seawater-anaesthetic solution while carefully removing any remaining leeches with forceps. All anaesthetised fish were returned to their holding aquarium to recover. The contents of each beaker and each 3 L tank was filtered through a 60 µm sieve and transferred separately to a glass petri dish for counting of parasites under the dissection microscope. The 60 µm mesh tank covers and tank sides were also inspected for any dislodged parasites. All *N. girellae* and *Z. arugamensis* were counted and preserved in vials of 70% ethanol.

Fish infected with *C. irritans* were placed into individual glass beakers containing seawater and a euthanasia concentration of 2-phenoxyethanol at 1.5 ml/L for 10 minutes (Neiffer and Stamper 2009). Thereafter, each fish was placed into a shallow glass Petri dish and *C. irritans* cells counted on the entire body surface under the dissection microscope at 20 X magnification. After counts of the body surface were made, all gill arches were excised to facilitate separate observations and counts. The seawater solution in each beaker, and experimental tank, and each 60 µm tank cover was similarly scrutinised for any dislodged *C. irritans* cells. All *C. irritans* cells were recorded per fish, and each individual fish and their gills was fixed in 10% phosphate-buffered formalin.

The same experimental setup was used for the environmental stage challenges excluding fish (Fig. 4.1.) I used 10 treatment and 10 handling control tanks per challenge. An average of 50 (30–88) *Neobenedenia girellae* eggs, 56 (30–131) *C. irritans* tomonts (cysts), or 88 (27–221) *Z. arugamensis* cocoons were introduced to both the treatment and the handling control tanks. These challenges ran for 12 hours diurnally or nocturnally as for the parasitic stage challenges, with the exception that the leech cocoon trial was completed over 24 hours, combining 12 simulated diurnal and nocturnal hours. At the end of each trial, the *N. girellae* eggs, *C. irritans* tomonts, or *Z. arugamensis* cocoons were counted under the dissection microscope.

4.3.5 Infection of fish with parasites

Cryptocaryon irritans infects the skin and gill tissue of its host fish. Adult trophont cells vacate the host and encyst (tomont stage) on the substrate in the environment where they undergo cell division to produce hundreds of reinfective, free-swimming microscopic theronts (Colorni and Burgess 1997). Tomont 'carpets' (cell aggregations forming a tomont monolayer; see Colorni and Burgess 1997) were collected from the glass substrate of the culture tank with a scalpel blade. These were washed in fresh seawater and incubated at 24°C and 35 ppt in a glass petri dish to ensure mass emergence of theronts for experimental infection using an artificial 12 hour day/night light regime. Theronts (~4 hours post-emergence, following Diggles and Lester 1996a) were then transferred to a beaker of fresh, filtered seawater (24°C and 35 ppt) via pipette and evenly distributed by continuous gentle aeration. Samples of the solution were taken with a pipette and observed on a haemocytometer. The theront-seawater solution was serially diluted using additional seawater to obtain a concentrated solution of 1000 cells per ml, following subsequent haemocytometer sample observations as required.

All fish for the *C. irritans* challenge experiments were placed into identical non-recirculating 3 L plastic aquaria containing gently aerated, fresh, filtered seawater (24°C and 35 ppt), and 1 ml of the theront-seawater solution was added via pipette to a final concentration of ~166 theronts/L. This concentration was used because theronts demonstrate a relatively low invasion success rate of between 5% and 20% (Matthews and Burgess 1995). Host fish and theronts were cohabited for 1 hour to allow successful infection (Colorni and Burgess 1997 *cf.* Diggles and Lester 1996a, b). Thereafter all individual fish were removed to identical non-recirculating 15 L plastic tubs containing gently aerated, fresh, filtered, seawater (24°C and 35 ppt) for 48 hours to allow trophonts to develop but not to mature (Colorni and Burgess 1997). Each fish was given a complete seawater exchange daily at the same temperature and salinity

to maintain water quality. After 48 hours post-infection, individual fish were removed and used for experimentation.

For the *N. girellae* challenge experiments, freshly laid embryonated eggs recovered from the culture were placed into a glass Petri dish containing fresh, filtered seawater, and hatched at room temperature (Brazenor and Hutson 2015). Thirty freshly hatched (<2 hours post-hatch, see Militz and Hutson 2015) free-swimming larvae (oncomiracidia) were removed via pipette and introduced to each identical non-recirculating 15 L plastic tub containing an individual fish, and gently aerated in fresh, filtered seawater (24°C and 35 ppt). Larval *N. girellae* were established and grown on fish for 144 hours prior to experimental use to avoid parasites reaching sexual maturity (Brazenor and Hutson 2015), but giving them enough time to grow to a size to facilitate easy observation and recovery. This was done to avoid any eggs being produced in the experimental system that could potentially hatch and reinfect fish. Over this period, each fish was given a complete seawater exchange daily at the same temperature and salinity to maintain water quality.

Leeches were collected by anaesthetising the host fish with 0.15 ml/L 2-phenoxyethanol and careful removal with a wet cloth. Only sub-adult leeches (~1 cm long) were used for experimentation. Fish were introduced to individual glass beakers of seawater containing 10 leeches each. On contact, leeches attached immediately, and fish were carefully transferred thereafter to the experimental or control tanks.

4.3.6 Preparation of environmental stages

Neobenedenia girellae produces large numbers of straw-coloured tetrahedral eggs which contain a tendril allowing them to attach to environmental structures. Egg embryonation can be observed by the development of pigmented eyespots in each developing oncomiracidium within the egg (Hutson *et al.* 2018; Fig. 4.1). Individual groups of embryonated eggs were

entangled on a ~1 cm² piece of fine bridal tulle using forceps (Fig. 4.1), and glued to a small glass petri dish for stability, prior to random allocation in the experiment.

Glass microscope slides were placed over the benthic surface area of both the *C. irritans* and *Z. arugamensis* culture tanks, allowing tomonts to encyst and therefore to attach, or cocoons to be cemented dorsally to one side, over 24 hours (Fig. 4.1). These slides were removed to the dissection microscope where they remain submerged in seawater, and the number of tomonts or cocoons were counted and recorded in pencil on each slide, prior to random allocation in the experiment.

4.3.7 Statistical analyses

All analyses were performed in R (Version 3.4.0; R Development Core Team 2017), using the packages 'glm2' (Marschner 2011) for generalised linear models, and 'car' (Fox and Weisberg 2011). Count proportions were used as the response variable for all analyses, i.e. the recovered proportion of the originally introduced amount of the parasitic (on fish) and the environmental stage of both parasite species to each of the treatment, and handling control tanks. Two approaches were used to explore the data for each parasite species, and data for each diurnal and nocturnal parasite experiment was run separately. First, I ran generalised linear models with a binomial regression and 'logit' link with '*shrimp species*' and '*treatment*' (shrimp presence *vs* shrimp absence), and the interaction '*shrimp* x *treatment*' as potential explanatory variables. These models indicated overdispersion. Therefore I re-ran the analyses with a quasibinomial regression and 'logit' link to account for overdispersion. This was done to detect whether an effect of treatment was significant, or not, in each model. Based on these results I explored pairwise comparisons for each separate shrimp species using '*treatment*' as the explanatory variable. I ran each of these as separate generalised linear models with a

quasibinomial regression and 'logit' link, analysed with Anova() in the 'car' package (see Appendix 3).

4.4 Results

Cleaner shrimp reduced parasite numbers on fish and their environmental stages under diurnal and nocturnal conditions (Figs 4.2–4.4), however shrimp species did not perform equally, and some species performed better at night. Two *L. vittata* were eaten by their clients during cohabitation at the completion of the nocturnal trials with *N. girellae* as the lights were turned on to recover the shrimp. This did not affect the result, and no other shrimp were lost during the study.

A treatment effect was detected in the initial exploratory diurnal and nocturnal statistical models for the *C. irritans* environmental stage [tomonts: $\chi^2_{(1,\, n = 80)} = 70.42$, $p < 0.001$] and nocturnal model parasitic stage [on fish: $\chi^2_{(1,\, n = 80)} = 176.91$, $p < 0.001$; $\chi^2_{(1,\, n = 80)} = 49.06$, $p < 0.001$], both the diurnal and nocturnal models for the parasitic stage [on fish: $\chi^2_{(1,\, n = 80)} = 48.99$, $p < 0.001$; $\chi^2_{(1,\, n = 80)} = 76.05$, $p < 0.001$], and the environmental stage [eggs: $\chi^2_{(1,\, n = 80)} = 10.59$, p 0.001; $\chi^2_{(1,\, n = 80)} = 33.38$, $p < 0.001$] for *N. girellae,* and the diurnal and nocturnal challenges for *Z. arugamensis* [$\chi^2_{(1,\, n = 60)} = 93.65$, $p < 0.001$; $\chi^2_{(1,\, n = 20)} = 60.24$, $p < 0.001$]. However, there was no treatment effect for the diurnal *C. irritans* parasitic stage [on fish: $\chi^2_{(1,\, n = 80)} = 0.16$, $p = 0.68$] model, suggesting that none of the shrimp species significantly reduced the parasitic stage of *C. irritans* during the day (Fig. 4.2a). Therefore, only models with a treatment effect were considered for further pairwise comparisons between treatment and control groups per shrimp species.

4.4.1 Cryptocaryon irritans

All shrimp species reduced *C. irritans* trophonts on infected fish nocturnally only (Fig. 4.2b). *Lysmata vittata* reduced trophonts by ~31.7% [$\chi^2_{(1,\, n\,=\,20)} = 11.51$, $p < 0.001$], followed closely by *L. amboinensis* and *U. antonbruunii* with a reduction of ~28.7% and 23.4% [$\chi^2_{(1,\, n\,=\,20)} = 11.56$, $p < 0.001$; $\chi^2_{(1,\, n\,=\,20)} = 48.59$, $p < 0.001$], respectively. *Stenopus hispidus* reduced trophonts by ~11.5% [$\chi^2_{(1,\, n\,=\,20)} = 11.4$, $p < 0.001$].

All shrimp reduced tomonts (non-parasitic stage) both diurnally and nocturnally, with the exception of *S. hispidus*, which only reduced tomonts diurnally (Fig. 4.2c, d). Shrimp species did not perform equally, and some showed preference for either diurnal or nocturnal performance (Fig. 4.2c, d). *Lysmata vittata* out-performed all other shrimp species, reducing the tomonts by ~69.4% diurnally [$\chi^2_{(1,\, n\,=\,20)} = 23.07$, $p < 0.001$; Fig. 4.2c] and 97.9% nocturnally [$\chi^2_{(1,\, n\,=\,20)} = 837.73$, $p < 0.001$; Fig. 4.2d]. *Lysmata amboinensis* reduced tomonts ~48.7% diurnally [$\chi^2_{(1,\, n\,=\,20)} = 18.93$, $p < 0.001$; Fig. 4.2c] and 75.3% nocturnally [$\chi^2_{(1,\, n\,=\,20)} = 35.92$, $p < 0.001$; Fig. 4.2d], while *U. antonbruunii* reduced tomonts diurnally by ~20.8% [$\chi^2_{(1,\, n\,=\,20)} = 5.13$, $p = 0.02$; Fig. 4.2c] and 46.8% nocturnally [$\chi^2_{(1,\, n\,=\,20)} = 15.04$, $p < 0.001$; Fig. 4.2d]. *Stenopus hispidus* reduced tomonts diurnally by ~17.5% [$\chi^2_{(1,\, n\,=\,20)} = 8.14$, $p = 0.004$; Fig. 4.2c].

4.4.2 Neobenedenia girellae

Only *L. amboinensis* and *U. antonbruunii* reduced *N. girellae* on infected fish both diurnally and nocturnally, while both *L. vittata* and *S. hispidus* only performed nocturnally. *Lysmata amboinensis* reduced infection by ~32% diurnally [$\chi^2_{(1,\, n\,=\,20)} = 7.25$, $p = 0.007$; Fig. 4.3a] and 34.6% nocturnally [$\chi^2_{(1,\, n\,=\,20)} = 13.37$, $p < 0.001$; Fig. 4.3b], followed closely by *U. antonbruunii* with diurnal, and nocturnal reduction of ~30% and 29.3% [$\chi^2_{(1,\, n\,=\,20)} = 6.81$, $p = 0.009$; $\chi^2_{(1,\, n\,=\,20)} = 10.92$, $p < 0.001$], respectively (Fig. 4.3a, b). *Lysmata vittata* and *S. hispidus* reduced

infection nocturnally by ~23.6%, and 23%, [$\chi^2_{(1,\, n = 20)} = 4.18$, $p = 0.04$; $\chi^2_{(1,\, n = 20)} = 8.21$, $p = 0.004$], respectively (Fig. 4.3a, b).

Both *Lysmata* species and *S. hispidus* reduced *N. girellae* egg numbers both diurnally and nocturnally (Fig. 4.3c, d). However, *U. antonbruunii* did not reduce egg numbers at all (Fig. 4.3c, d). *Lysmata vittata* reduced egg numbers by ~74.4% and 86.1% [$\chi^2_{(1,\, n = 20)} = 36.17$, $p < 0.001$; $\chi^2_{(1,\, n = 20)} = 50.64$, $p < 0.001$], diurnally and nocturnally, respectively (Fig. 4.3c, d). This was followed by *L. amboinensis*, which reduced egg numbers by ~49% diurnally [$\chi^2_{(1,\, n = 20)} = 13.05$, $p < 0.001$; Fig. 4.3c] and 79% nocturnally [$\chi^2_{(1,\, n = 20)} = 27.36$, $p < 0.001$; Fig. 4.3d]. *Stenopus hispidus* reduced eggs by ~35.9% diurnally and 28.8% nocturnally [$\chi^2_{(1,\, n = 20)} = 9.3$, $p = 0.002$; $\chi^2_{(1,\, n = 20)} = 6.54$, $p = 0.01$], respectively (Fig. 4.3c, d).

4.4.3 Zeylanicobdella arugamensis

Some leeches vacated the host fish during experimentation and were recovered from the sides of the treatment and control tanks. Analyses were performed on the combined numbers of leeches recovered from the fish and tank surfaces to provide a result for overall leech reduction by shrimp (Fig. 4.4a). *Lysmata amboinensis* reduced leeches by ~65% diurnally [$\chi^2_{(1,\, n = 20)} = 29.81$, $p < 0.001$; Fig. 4.43a], and by ~77% nocturnally [$\chi^2_{(1,\, n = 20)} = 60.24$, $p < 0.001$; Fig. 4.4a]; *S. hispidus* reduced leeches on fish diurnally by ~74% [$\chi^2_{(1,\, n = 20)} = 39.44$, $p < 0.001$; Fig. 4.4a]; *L. vittata* reduced numbers of leeches by ~25% [$\chi^2_{(1,\, n = 20)} = 19.4$, $p < 0.001$; Fig. 4.4a], and leech cocoons over 24 hours by ~97% [$\chi^2_{(1,\, n = 20)} = 265.95$, $p < 0.001$; Fig. 4.4b]. A performance matrix was constructed based on the performance of each shrimp species, against all parasite species (Fig. 4.5). This indicated that *L. vittata* out-performed all other shrimp species in consuming parasites' environmental stages.

4.5 Discussion

Parasites in high numbers can cause mortalities in fish populations. Visible parasites, and the physical damage caused by them, can result in direct rejection by consumers, which can be costly to the aquaculture farmer, wholesaler and retailers (Ogawa 1994). These problems are often mitigated at farm-level through targeted applications of chemical therapies (Aaen *et al.* 2015). These practices contribute additional negative consumer sentiment (Banarjee *et al*. 2014; Claret *et al.* 2014). In response to this, appropriate biocontrols could reduce both the frequency of chemical interventions, and the parasites that result in unsightly damage.

This study is the first to demonstrate the potential of four cleaner shrimp species as biocontrols against three economically important fish parasite species in aquaculture, and supports peppermint shrimp *L. vittata* as the first candidate species for further testing under aquaculture conditions. Cleaner shrimp may offer superior benefits to traditionally-used cleaner fishes as biocontrols as they are also capable of reducing parasite reinfection pressure directly by consuming environmental life-stages which are resistant to chemical therapies (Militz and Hutson 2015). Employing these shrimp to reduce environmental life-stages also implies that no direct contact between shrimp and client fish is needed, and therefore shrimp predation risk is minimised. Additionally, shrimp are not susceptible to the ectoparasites of fishes they would be employed to control, which has become a recent concern with cleaner fishes (Karlsbakk *et al.* 2013, 2014, Karlsbakk 2015; Haugland *et al.* 2017; Powell *et al*. 2017).

An arguable limitation to using cleaner shrimp is their availability and cost. However, recent technological advances have supported successful captive breeding of cleaner shrimp species (Hettiarachchi and Edirisinghe 2016). *Lysmata vittata,* which out-performed all other shrimp species examined in this study, is currently produced commercially in Australia for the ornamental trade, and the cost per unit is based on the scale of demand. This shrimp has a large geographic distribution in the Asia-Pacific region, and through parts of the Indo-Pacific and

North to Russian waters (Marin *et al*. 2012). This distribution range incorporates some of the major sub-tropical and tropical aquaculture-producing nations (Asia-Pacific Fishery Commission 2014), including China, Philippines, Japan, Indonesia, and Australia (Marin *et al*. 2012). Using *L. vittata* as a biocontrol in aquaculture offers a sustainable alternative to chemical interventions to treat fish parasites, and may improve the overall sentiment of consumers regarding farmed fish.

I was able to test the performance of cleaner shrimp by maintaining three parasite cultures *in vivo* in the laboratory. The results show that different cleaner shrimp species vary in their cleaning performance. All four shrimp species tested in this study reduced the ciliate and monogenean on the fish directly (Figs 1a, b, 2a, b, 4). However, both *L. vittata* and *S. hispidus* were strictly nocturnal when removing these parasite species off the fish (Figs 1b, 2b, 4), while both *L. amboinensis* and *U. antonbruunii* reduced monogeneans on fish diurnally and nocturnally (Figs 2a, b, 4). None of the four shrimp species reduced ciliates on the fish diurnally (Figs 1a, 4). This may simply indicate a shrimp preference for nocturnal removal of this parasite only, a difference in the host behaviour, or behavioural changes of the parasite itself on the fish, making it more susceptible to predation nocturnally. The experiment used two day old trophonts at 24°C, and I did not recover any protomonts or tomonts in the experimental tanks, which concurs with results of these previous studies. This indicates that trophonts were not yet vacating the host in the experiment. Some leeches did vacate the host during the challenges. I tried to avoid this by specifically selecting sub-adult leeches, but this behaviour likely represents a normal strategy after leeches have become satiated with a blood meal (Kearn 2007). The numbers of leeches were limited so I completed diurnal and nocturnal challenges for one shrimp species (*L. amboinensis*), diurnal challenges for two (*L. vittata* and *S. hispidus*), and a 24 hour environmental stage (cocoons) challenge for *L. vittata* only (Fig. 3). *Lysmata*

amboinensis reduced leeches both diurnally and nocturnally, while *L. vittata* and *S. hispidus* reduced leeches diurnally (Figs 3a, 4).

Not all shrimp reduced the environmental stage of ciliates and monogeneans (Figs 1c, d, 2c, d, 4). *Urocaridella antonbruunii* did not reduce the number of monogenean eggs either diurnally or nocturnally (Figs 2c, d, 4), suggesting that this shrimp species does not consume monogenean eggs. However, *U. antonbruunii* did reduce ciliate tomonts in the environment, and showed a better nocturnal than diurnal performance (Figs 1c, d, 4). Why this shrimp species would consume *C. irritans* tomonts and not monogenean eggs may be a consequence of their inability to masticate the latter, which have a hard protective sclerotin shell. Therefore, unlike the other three shrimp species, *U. antonbruunii* may be predisposed to consuming softer prey items. *Stenopus hispidus* reduced monogenean eggs diurnally and nocturnally (Figs 2c, d, 4), but was the only shrimp species that did not consume ciliate tomonts nocturnally (Figs 1d, 4). Both *Lysmata* species demonstrated the highest level of environmental stage reduction for ciliates and monogeneans, with an increased performance nocturnally (Figs 1c, d, 2c, d, 4). *Lysmata vittata* reduced leech cocoons over the 24 hour period (Figs 3b, 4). Overall, it was the best performer of parasite environmental stage reduction (Figs 1c, d, 2c, d, 4).

It was evident from the results of the environmental stage challenges that individual shrimp of the same species did not perform equally. The same phenomenon was previously observed for *L. amboinensis* (Militz and Hutson 2015). Similarly *S. hispidus* was discussed in terms of different individual responses to environmental cues (Esaka *et al*. 2016). This phenomenon likely reflects shrimp foraging in an area-restricted search pattern, where the shrimp consume as much as they can on each chance encounter with a prey item. Historically, the legitimacy of *S. hispidus* as a cleaner shrimp had been questioned (Bunkley-Williams and Williams 1989; McCammon *et al.* 2010). In the first laboratory-based empirical study on cleaner shrimp, *S. hispidus* did not remove the parasitic isopod *Anilocra haemuli* Williams and

Williams, 1981 off host fish (Bunkley-Williams and Williams 1989). However, in a semi-natural microcosm, *S. hispidus* did appear to have a preference for consuming larger individual *N. melleni* monogeneans, although it did not reduce parasite numbers on hosts (McCammon *et al*. 2010). My data indicate that this shrimp species significantly reduced ciliates, monogeneans and leeches on host fish. Apparent difference in the capacity of *S. hispidus* to reduce the closely related monogeneans *N. girellae* in the present study and *N. melleni* in the previous macrocosm study (McCammon *et al*. 2010), is unlikely due to parasite species differences. Fish in the macrocosm were subjected to incoming water from an exhibit with a known infection of *N. melleni* continuously for at least 14 days (McCammon *et al.* 2010), and would therefore have likely been repeatedly infected over this period. From my data, *S. hispidus* demonstrated a nocturnal bias towards removal of ciliates and monogeneans off host fish. This supports the earlier conclusions, that *S. hispidus* is primarily nocturnal (Collette and Talbot 1972; Corredor 1978; Jonasson 1987; McCammon *et al.* 2010). However, *S. hispidus* does clean fishes and turtles diurnally (Sazima *et al*. 2004a), a behaviour which my results also support for the reduction of leeches. Diurnal cleaning by *S. hispidus* was suggested to be a function of changing light conditions (Esaka *et al.* 2016), but it appears that *S. hispidus* may prefer to prey on different fish parasites diurnally or nocturnally, which may reflect more the behaviour of the clientele that have these parasites.

Urocaridella antonbruunii is the first *Urocaridella* species reported to reduce monogeneans. Although a similar study found that *Urocaridella* sp. c (yellow-beaked cleaner shrimp) eat dead *Benedenia* sp. monogeneans offered to them, only *Ancylomenes holthuisi* (Bruce, 1969) was used to test shrimp efficacy at removing this monogenean on *Ctenochaetus striatus* (Quoy and Gaimard, 1825) (see Becker and Grutter 2004). The same shrimp, *U*. sp. c, was also used to evaluate hunger levels on cleaning time, using *Cephalopholis cyanostigma* (Valenciennes, 1828) infected with *Benedenia* sp. monogeneans (Becker and Grutter 2005).

Therefore *U.* sp. c likely also consumes monogeneans, at least of the family Capsalidae. Currently, temperate and tropical cleaner shrimp that eat monogeneans represent the families Hippolytidae, Palaemonidae, and Stenopodidae.

Cleaner shrimp may provide a different type of cleaning service than that of cleaner fishes (Titus *et al*. 2015). This is supported by an apparent 'cleaning structure discordance', evident by an apparent lack of competition between cleaner fish and shrimp observed in the wild, and a difference in temporal interactions with the same clients (Titus *et al.* 2015). The specific cleaning function of shrimp remains largely unresolved, driven by underexplored proximate causes (Becker and Grutter 2005; Chapter 2). My data suggest that many cleaner shrimp may likely offer the combined benefit of a symbiotic cleaning service, and non-symbiotic cleaning as a by-product benefit in the shelters shared by their resting clients, which probably reduces parasite reinfection success. Parasites have different life-cycle and reproductive strategies. For example, some monogeneans release eggs specifically following the onset of darkness (Mooney *et al.* 2008) or produce more eggs at night (Dinh Hoai and Hutson 2014), and the ciliate *C. irritans* vacates the host at night. Many parasites have infective stages that also emerge nocturnally, e.g. *C. irritans* (see Matthews and Burgess 1995). Cleaner shrimp appear to actively reduce parasites nocturnally, and may therefore be an important source of parasite control on a reef at night when diurnal fish cleaners, like *Labroides dimidiatus*, are inactive.

Further research into the potential use of cleaner shrimp in aquaculture is warranted. A recent study estimated the global diversity of cleaner shrimp species to be approximately 51 known species from 11 genera, representing six families (Vaughan *et al.* 2016; Chapter 2). These taxa include tropical, sub-tropical and temperate marine representatives. Further work could explore whether client fish species recognise cleaner shrimp species as cleaners under aquaculture conditions to determine whether direct cohabitation, as is used in salmon farming

with cleaner fishes, is feasible with shrimp. However, I have demonstrated that cohabitation is not necessary in reducing parasite problems. The advantages of exploiting shrimps' natural predatory behaviour of parasites and other pathogens, particularly their environmental stages, implies a wealth of potentially new solutions to existing and future aquaculture health problems.

Acknowledgements

I am grateful to Emeritus Professor Rhondda Jones for her statistical advice, to Dr Derek Sun for assisting with the collection of *Urocaridella antonbruunii*, and to Dr Richard Knuckey for the generous contribution of the experimental grouper used in this study.

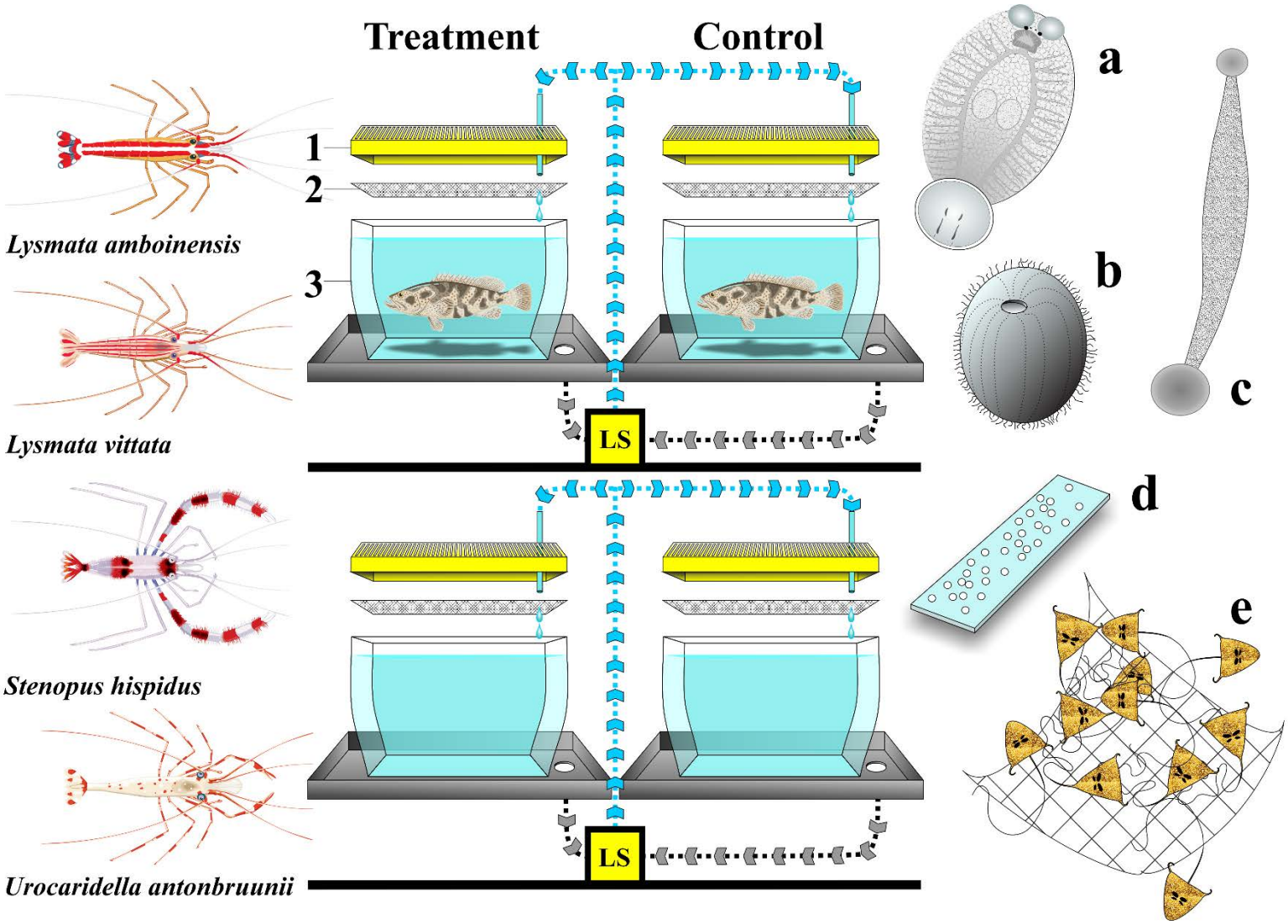

Fig. 4.1. Experimental design demonstrating treatment and control setup for a single replicate for parasitic (on fish) and environmental stages, and shrimp species used. **a.** *Neobenedenia girellae*; **b.** *Cryptocaryon irritans* trophonts; **c.** *Zeylanicobdella arugamensis*; **d.** *Cryptocaryon irritans* tomonts (cysts) or *Z. arugamensis* cocoons on microscope slide; **e.** Embryonated *Neobenedenia girellae* eggs attached to bridal tulle; LS. Recirculating seawater life-support system with filtered influent (blue arrows), and effluent (grey arrows) schematic water flow; 1. Fitted tank lid; 2. 60 µm mesh tank cover; 3. Individual experimental tank positioned inside water catchment tray.

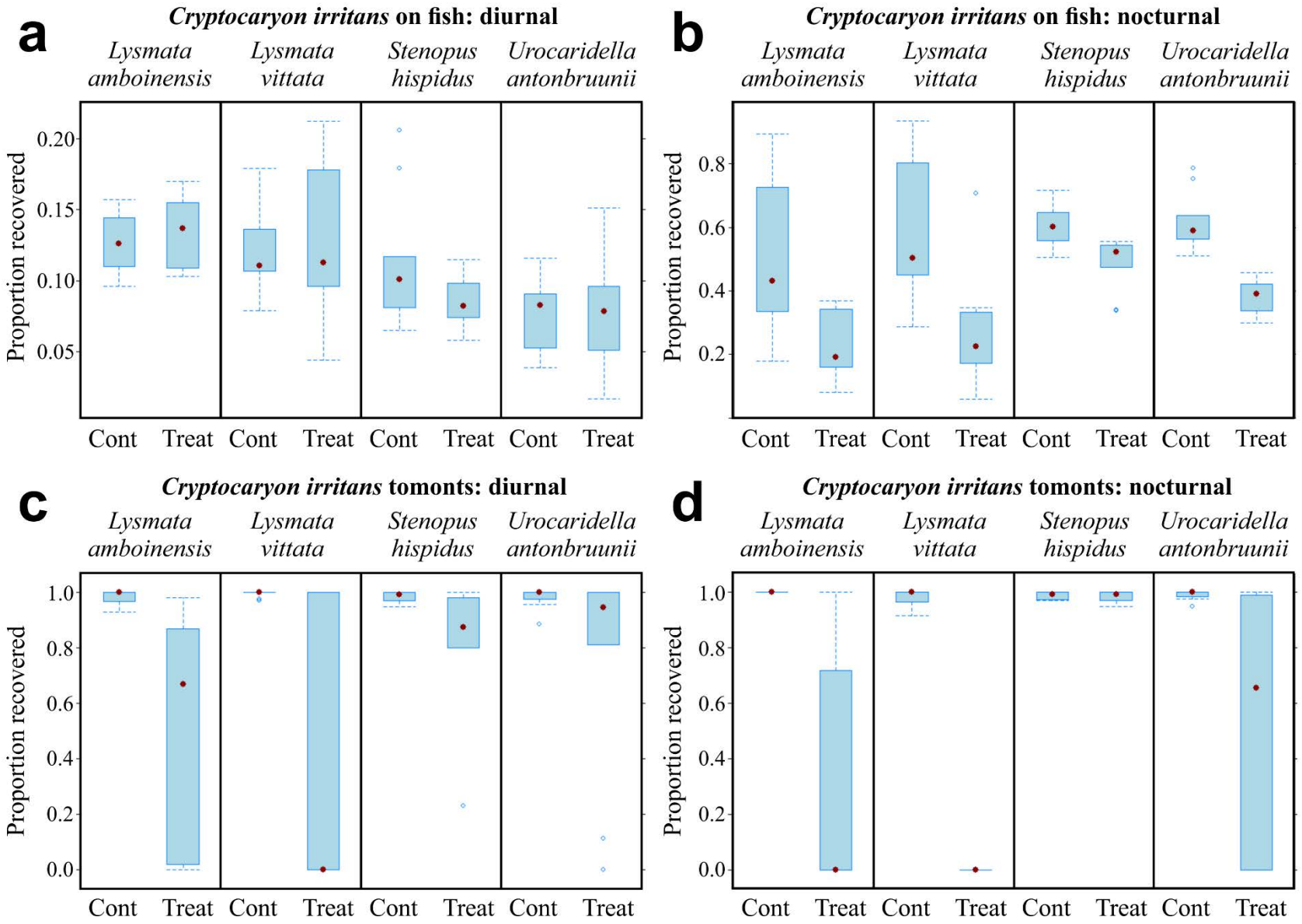

Fig. 4.2. Effect of cleaner shrimp on *Cryptocaryon irritans*. **a.** Reduction of *C. irritans* trophonts (parasitic stage) on fish by cleaner shrimp diurnally; **b.** Nocturnally; **c.** Reduction of *C. irritans* tomonts (environmental stage) diurnally; **d.** Nocturnally. Data expressed as the proportion recovered. Ten observations per condition.

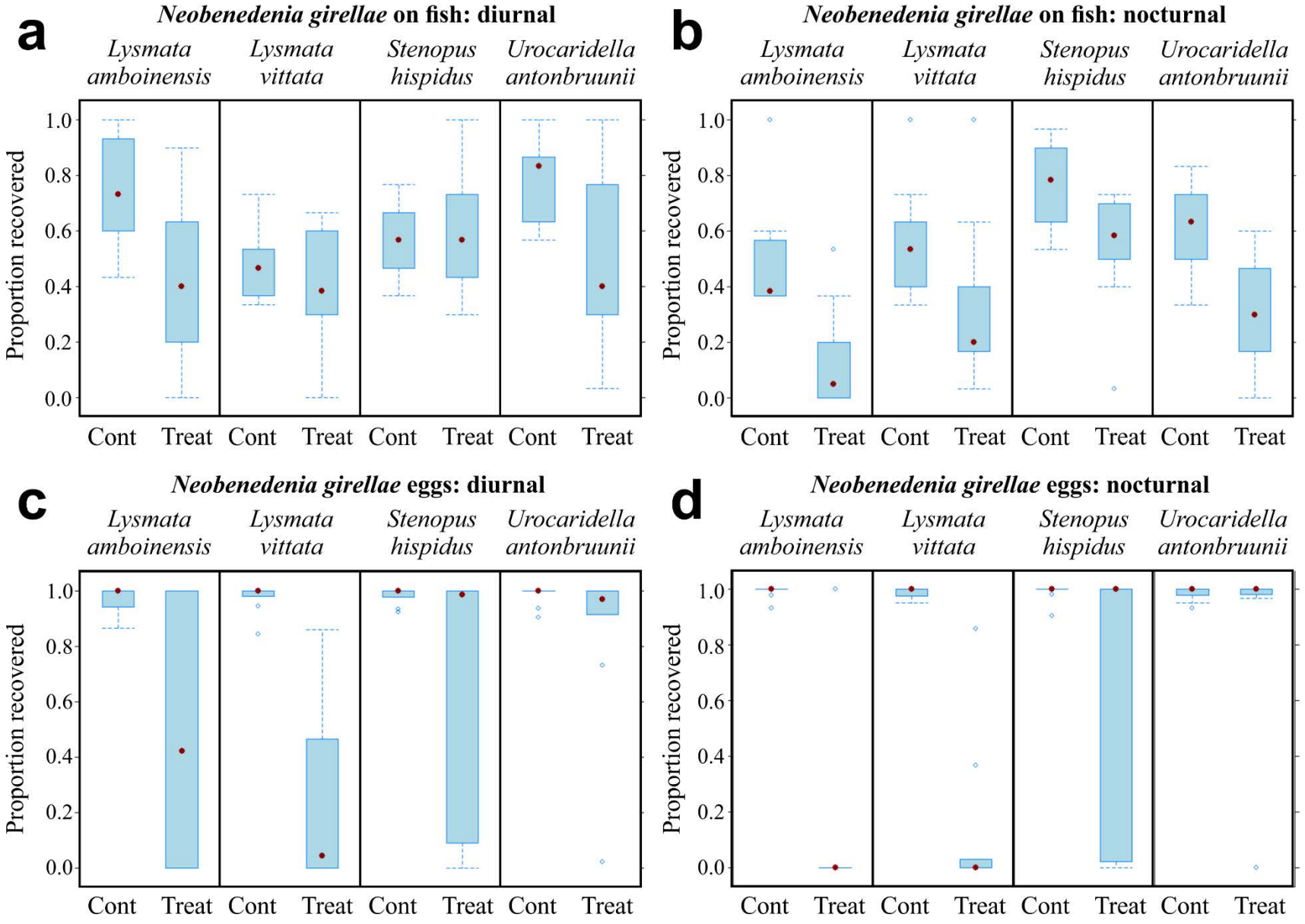

Fig. 4.3. Effect of cleaner shrimp on *Neobenedenia girellae*. **a.** Reduction of sub-adult *N. girellae* (parasitic stage) on fish by cleaner shrimp diurnally; **b.** Nocturnally; **c.** Reduction of *N. girellae* eggs (environmental stage) diurnally; **d.** Nocturnally. Data expressed as the proportion recovered. Ten observations per condition.

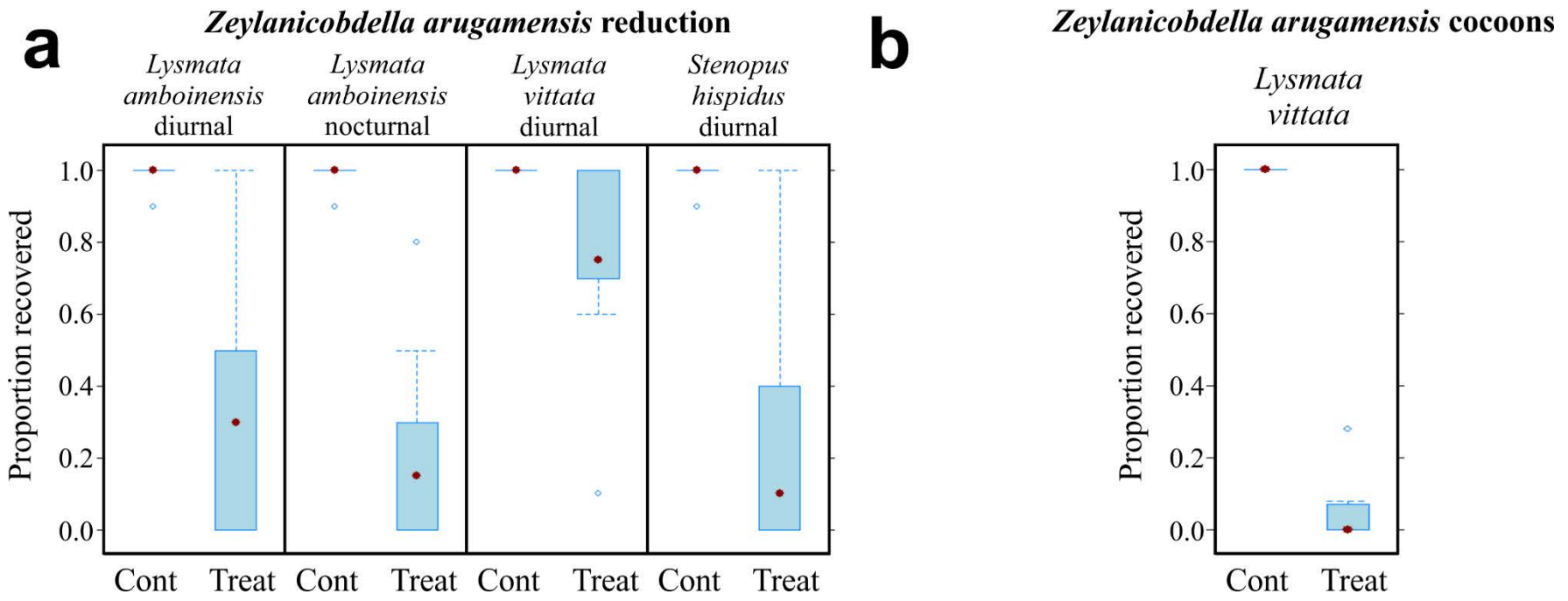

Fig. 4.4. Effect of cleaner shrimp on *Zeylanicobdella arugamensis*. **a.** Reduction of sub-adult *Z. arugamensis* (parasitic stage) by cleaner shrimp diurnally or nocturnally; **b.** Reduction of *Z. arugamensis* cocoons (environmental stage) by *Lysmata vittata* over 24 hours. Data expressed as the proportion recovered. Ten observations per condition.

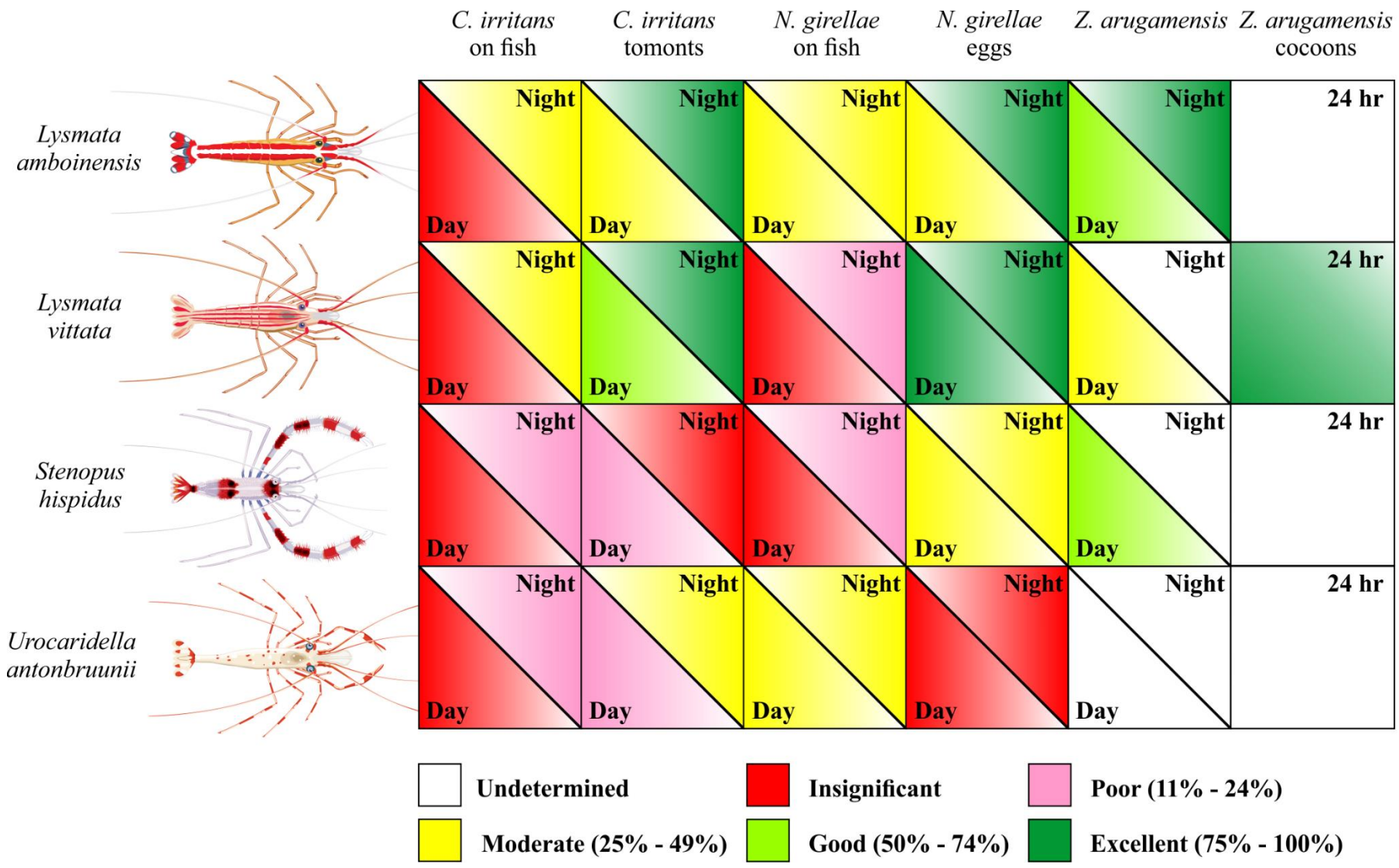

Fig. 4.5. Shrimp species performance matrix. Ranking in the ability of each shrimp to remove parasites: Undetermined, no current data; Insignificant, $p > 0.05$; Poor, 11%–24% reduction; Moderate, 25%–49% reduction; Good, 50%–74% reduction; Excellent, 75%–100% reduction. Ten observations per cell.

CHAPTER 5

Cleaner shrimp remove parasite eggs on fish cages

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In this chapter, I test the ability of the selected candidate shrimp species, *Lysmata vittata*, from Chapter 4 to perform as a biocontrol under recirculating aquaculture conditions to address the fourth thesis aim.

5.1 Abstract

Benthic stages of cultured fishes' ectoparasites are a major contributor to persistent reinfections in aquaculture. These stages tend to be resistant to chemical therapies and are costly in time and labour to manage. Cleaner shrimp, unlike cleaner fishes, prey on benthic stages, indicating they have the potential to reduce parasite reinfection pressure without having to be in direct contact with the client fish. Cleaner shrimp have never been used as biocontrols in commercial aquaculture, but offer an advantage over cleaner fishes, because they are not susceptible to the ectoparasites of their clients. I present the first investigation of a cultured cleaner shrimp, *Lysmata vittata*, as a biocontrol agent against the eggs of the economically important

cosmopolitan ectoparasite *Neobenedenia girellae* infecting cultured juvenile grouper, *Epinephelus lanceolatus*, under simulated recirculating aquaculture conditions. *Lysmata vittata* removed the eggs of *N. girellae* entangled on the mesh of the culture cages and significantly reduced *N. girellae* recruitment on fish by ~87%. My results demonstrate the value of cleaner shrimp in addressing ectoparasite problems, and highlight the importance of investigating novel biocontrol strategies in aquaculture.

5.2 Introduction

Biocontrols are living organisms used to suppress the density of a pest organism's population, or its associated impact, rendering it less abundant and less problematic (Eilenberg *et al*. 2001). However, where the targeted pest organism is parasitic or pathogenic, it is critical to select appropriate biocontrol agents that are not susceptible, and which do not pose a risk of enhancing pathogen virulence (*cf*. Madhusudana Rao and Lalitha 2015).

Biocontrol use in marine environments remains largely underexplored, and is in a current stage of infancy (Atalah *et al.* 2015). Aquaculture consideration of, and the use of biocontrols against pathogenic agents has focused largely on the use of microbial control strategies, e.g. probiotics, bacteriophages, and specific predatory bacteria to target economically important bacterial finfish and shellfish diseases (see examples in Verschuere *et al*. 2000; Cao *et al.* 2014; Madhusudana Rao and Lalitha 2015). To date, the only biocontrol application against fish ectoparasites in commercial aquaculture has been the use of cleaner fishes (wrasses, *Centrolabrus exoletus* (Linnaeus, 1758), *Ctenolabrus rupestris* (Linnaeus, 1758), *Labrus bergylta* Ascanius, 1767, *Symphodus melops* (Linnaeus, 1758), and *Cyclopterus lumpus* Linnaeus, 1758) to control sea lice (*Lepeophtheirus salmonis* (Krøyer, 1837) and *Caligus elongatus* von Nordmann, 1832), parasitic on Atlantic salmon (*Salmo salar* Linnaeus,

1758) and other salmonids farmed in marine waters in Europe (Deady *et al*. 1995; Treasurer 2002; Skiftesvik *et al*. 2013; Leclercq *et al.* 2014; González and de Boer 2017).

The control of sea lice by cleaner fishes follows an augmentative biocontrol approach, which involves the introduction of indigenous natural predators to control pest organisms (see Atalah *et al.* 2015). This strategy offers clear benefits in salmon farming by reducing numbers of reproductive adult sea lice. However, the success of this type of biocontrol strategy relies primarily on the feeding preferences of the biocontrol agents (Hajek 2004; Atalah *et al.* 2015). The utility of the cleaner fishes' model, notably wrasses in Europe had traditionally been supported by the combination of a specific feeding preference of the selected cleaner species for the few problematic sea lice species (see González and de Boer 2017), and little overlap of their known parasite diversity with that of the cultured salmon (Treasurer 2012). Nonetheless, recent evidence suggests that cleaner fishes, including lumpfish are susceptible to other more generalist pathogens important to salmon and other fishes, including *C. elongatus*, and *Paramoeba perurans* (Young, Crosbie, Adams, Nowak & Morison, 2007) *sensu* Feehan *et al.* (2013), the aetiological agent of amoebic gill disease (Karlsbakk *et al.* 2013, 2014; Karlsbakk 2015; Haugland *et al.* 2017; Powell *et al*. 2017). This demonstrates a clear risk of using a cleaner fishes' model against the pathogens of other fishes, but also the limited scope for using these cleaner fishes against other host-parasite models and in other geographical regions.

The Asia-Pacific region produces the majority of the world's aquaculture products, yet no biocontrol use is employed against the ectoparasites of farmed fishes in this region. Recently, Shinn *et al.* (2015) estimated aquaculture stock losses in parts of Asia to be between 30% and 50% as a result of parasitic agents, excluding viruses and bacterial pathogens. The diversity of economically important ectoparasites of cultured marine finfish listed by Shinn *et al.* (2015) for this region is high, represented by many protozoans and metazoans with a direct life-cycle, many of which have a wide distribution range and low host-specificity (see Shinn *et*

al. 2015). It is therefore unlikely that any cleaner fishes would offer a viable option for ectoparasite biocontrol in tropical finfish aquaculture. However, a currently unexplored and potentially viable alternative may be the use of cleaner shrimp in a similar augmentative biocontrol approach.

There are an estimated 51 cleaner shrimp species known globally (Vaughan *et al*. 2016; Chapter 2) that interact naturally with various client species, of which the majority are marine teleosts. Many cleaner shrimp species directly remove and consume the ectoparasites in a density dependent manner (e.g. Becker and Grutter 2005) from their clients through repetitive symbiotic cleaning interactions and some species are also known to prey on the environmental (benthic) stages of the ectoparasites (e.g. Militz and Hutson 2015; Chapter 4). In so doing, these shrimp can reduce the reinfection pressure on host fishes (Militz and Hutson 2015; Chapter 4). No cleaner shrimp species is known to be susceptible to the ectoparasites of marine fishes, which reflects the co-evolved host-specificity of these fish ectoparasites (Poulin 1995), and a subsequent advantage that cleaner shrimp may offer over cleaner fishes in finfish aquaculture. Cleaner shrimp have never been used as a biocontrol agent against fish ectoparasites in commercial aquaculture. However, the gregarious rock shrimp (*Rhynchocinetes typus* H. Milne Edwards, 1837), a non-cleaner species, was used successfully to reduce biofouling of suspended scallop cultures by Dumont *et al*. (2009). This is the only example of a shrimp being used as a biocontrol in aquaculture, and benefits included a reduced mortality and increased growth of the farmed scallops (Dumont *et al*. 2009).

A large contributor to the ectoparasite problems in aquaculture is the resilience and sheer volume of the benthic stages of the different parasite species. These eggs, cocoons and cysts remain attached to culture cages and other farm infrastructure, and ultimately hatch or release their reinfective stages to infect farm stock in high numbers. A prime example is *Neobenedenia girellae* (Hargis, 1955), a cosmopolitan monogenean fluke ectoparasite of

serious economic concern throughout the Indo-Pacific region (Brazenor *et al*. 2018), which is responsible for morbidity and mortality in a diversity of cultured marine fishes including members of Carangidae, Cichlidae (marine acclimated tilapias), Lateolabracidae, Latidae, Paralichthyidae, Rachycentridae, Serranidae, and Tetraodontidae (Ogawa *et al*. 1995; Brazenor and Hutson 2015; Shirakashi and Hirano 2015; Shinn *et al.* 2015; Brazenor *et al.* 2017a). Acute infections of farmed fish with *N. girellae* result in severe mortality events, with fish subjected to stressful conditions or naïve stock without prior-acquired immunity most at risk (Deveney *et al*. 2001; Shirakashi and Hirano 2015). The traditional control measure for *N. girellae* eggs on fish cages remains the frequent cyclic replacement of contaminated nets with disinfected nets, which is largely ineffective, and which contributes to labour time and cost (Shirakashi and Hirano 2015).

I selected the cleaner shrimp *Lysmata vittata* (Stimpson, 1860) for testing as the first cleaner shrimp biocontrol candidate under aquaculture conditions, based on its superior performance at benthic parasite stage reduction in a series of previous laboratory trials (Chapter 4). In the present study, I aimed to test the efficacy of *L. vittata* against the benthic egg stage of *N. girellae* and subsequent infection on farmed grouper, *Epinephelus lanceolatus* (Bloch, 1790) kept in oyster mesh net cages under simulated recirculating aquaculture conditions.

5.3 Methods

5.3.1 Animal ethics and welfare

Ethical approval was granted prior to commencement of this study under the James Cook University Ethics Committee Permit number A2260, conforming strictly to the national regulations set out in the National Health and Medical Research Council (2013) Australian code for the care and use of animals for scientific purposes, 8th edition, under Section 39 of the National Health and Medical Research Council Act, 1992. Fish were subjected to temporary

infection by the ectoparasite *N. girellae*. As part of the experiment, freshwater bathing for 5 minutes using dechlorinated tap water was employed to kill and to dislodge 100% of these ectoparasites (Kaneko *et al*. 1988) for recovery and counting, and is a routine method used in aquaculture to control ectoparasites (Hutson *et al*. 2018).

5.3.2 Animals and experimental design

Four hundred and eighty juvenile *E. lanceolatus* from a single cohort (~150 mm in total length) were donated for my research by a commercial grouper hatchery in Cairns, Queensland, Australia. All fish were initially given a 5 minute freshwater bath with dechlorinated tap water on arrival before being quarantined together in the commercial trials laboratory of the Marine Parasitology Laboratory (MPL), James Cook University (JCU) for 30 days on a dedicated marine recirculating life-support system. One hundred and twenty commercially produced peppermint cleaner shrimp (*L. vittata*), also of a single cohort, were purchased from a commercial producer in Tasmania, Australia, and shipped to us once they had reached adulthood (~30 mm in total length). On arrival, all cleaner shrimp were quarantined for 30 days in a separate, isolated recirculating system. During the quarantine period and the experiment, all fish were fed daily to satiation with Ridley Aquafeed marine float commercial marine fish pellets, and the cleaner shrimp were fed daily with defrosted, commercially available *Mysis* sp. shrimp.

The commercially important monogenean *N. girellae* is continuously cultured in the separate MPL culture facility at JCU (see Hutson *et al*. 2018). Prior to experimentation, freshly laid *N. girellae* eggs were isolated from the culture and incubated at 24 ºC in a large glass Petri dish containing fresh, filtered seawater (salinity = 35 ppt). Eggs were monitored daily under a Leica M60 dissection microscope for embryonic development and hatching (see Hutson *et al*. 2018). Free-swimming larvae (oncomiracidia) hatched on day four (see Brazenor and Hutson

2015) and were collected via pipette and counted before immediately being transferred to a glass beaker of fresh, filtered seawater for the experiment.

All fish were transferred to a circular 500 L tank containing fresh, pre-filtered seawater supplied with continuous aeration through an air diffuser. The glass beaker containing ~10 000 fresh viable oncomiracidia was carefully introduced to the tank of fish, with care to distribute the contents as evenly throughout the tank as possible while maintaining continuous aeration. Fish were cohabited with the oncomiracidia for 1 hour. After an hour, individual fish were netted out using a soft aquarium hand-held net and were randomly assigned to eight identical separate 500 L circular tanks containing an inner plastic oyster mesh cage (1 m diameter; mesh size = 7 mm diameter), representing four treatment and four control replicates (i.e. 60 fish in each cage). These four treatment and four control tanks received constant recirculating aerated and biologically-filtered seawater. In addition, seawater was recirculated through an algae scrubber containing live, growing *Caulerpa taxifolia* (M.Vahl) C. Agardh, 1817 for nitrate export. No UV disinfection, or foam fractionation was employed, and no seawater exchanges were performed during the experiment. Seawater conditions (Fig. 5.1) were monitored daily with a Hach hand-held temperature and dissolved oxygen meter, a standard refractometer, a Eutech Scan2 pH meter, and ornamental aquarium nitrogenous waste test kits. Artificial light (cool white fluorescent overhead lighting) was maintained on a 12:12 hr light:dark regime.

To establish parasite egg biofouling on the experimental cages, *N. girellae* were allowed to develop on the fish to sexual maturity (7 days post-infection; Brazenor and Hutson 2015), and an additional 2 days to allow at least 3 consecutive days' egg production according to the biological data of Brazenor and Hutson (2015) at 26 ºC and 35 ppt salinity. On day 9 post-infection all fish were removed from their oyster mesh cages and given a 5 minute freshwater bath in dechlorinated tap water to kill and to remove adult *N. girellae* (see Kaneko *et al.* 1988), and therefore to cease further egg production, before being returned to their

original cages. An average (± SE) intensity of 18.7 ± 2 (11–32) adult *N. girellae* per fish, representing ~90% initial infection success, was calculated from a sample of 10, and considered benign for similar-sized hosts by Deveney *et al*. (2001). Immediately after the fish were returned to their cages 30 individual adult *L. vittata* were introduced to each of the four treatment tanks to patrol the outside of the oyster mesh cages (Fig. 5.2).

Twenty fish were sampled randomly from each cage on consecutive days 11, 12, and 13 post-infection, corresponding with hatching of the eggs at 26 ºC and 35 ppt salinity, and subsequent recruitment (Brazenor and Hutson 2015), and were individually given a 5 minute freshwater bath using separate plastic containers of dechlorinated tap water. The contents of each bath was filtered through a 23 μm sieve, decanted into separate labelled sample jars and preserved in 70% ethanol for subsequent counting of juvenile parasites. After their freshwater bath, all fish were introduced to a separate recirculating marine life-support system to recover. There were no fish or shrimp mortalities during the experiment.

Each sample jar was emptied into a large glass Petri dish, and its contents inspected under the Leica dissection microscope. All individual *N. girellae* parasites were collected via pipette, manually counted, and preserved in separate, labelled vials of 70% ethanol. This subsequent analytical process took four months to complete.

5.3.3 Statistical approach

I used mixed effects random-intercept models to analyse the parasite count data over the three sampling days, providing the resolution to optimise data modelling to density dependent predation of the cleaner shrimp, while accounting for repeated sampling from experimental tanks. Generalised linear regression was not required because parasite count data which consisted of predominantly high counts and no zeros, when log-transformed, produced normally-distributed residuals. In addition, the mixed effects random intercept models were

more applicable to account for different levels of residual variation in the response variable (*log of parasite counts*) after log-transformation (e.g. between days and between treatment). Water quality data were separately analysed over the entire experiment using a standard linear regression. All analyses were performed in R (Version 3.4.0; R Development Core Team 2017). Mixed effects random-intercept models were produced using the package 'nlme' (Pinheiro *et al.* 2018). All models passed diagnostic scrutiny. I constructed three mixed effects random-intercept models; two with correlation of variance structures for variance differences in treatment, or treatment-day combination groups, and one without a correlation of variance structure (see Appendix 4). These models were then compared using the anova() function, and the model accounting for correlation of variance structures for the treatment-day combination was considered the most improved model for the data (see Appendix 4). The improved model tested the *log of parasite counts* (the response variable) as a function of *Treatment* (with or without shrimp) and *Day* (the fixed effects), using the interaction terms *Treatment* x *Day*, and *Tank* as the random effect (see Appendix 4).

5.4 Results

Lysmata vittata consumed *N. girellae* eggs entangled on the oyster mesh fish cages (Fig. 5.3) and subsequently reduced the number of *N. girellae* recruitment by ~87% [ANOVA: $F_{1,6}$ = 173.36, p <0.0001; Fig. 5.4]. An average (± SE) of 964.2 ± 77.4 (101–9,851), and 123.4 ± 3.5 (12–350) *N. girellae* post-larvae were recovered from fish in the control and treatment groups, respectively. Numbers of *N. girellae* on fish across the experiment decreased with time by ~37% by day 3 [ANOVA: $F_{2,468}$ = 31.20, p <0.0001; Fig. 5.4]. The regression results for the fixed effects are presented in Table 5.1. Water quality parameters were not statistically different between treatment groups [ANOVA: $F_{1,24}$ = 1.27, p = 0.27], and remained stable for the

duration of the experiment (see Fig. 5.1). Temperature and salinity remained at ~26 ºC, and ~35 ppt.

5.5 Discussion

My results demonstrate for the first time the potential of the cleaner shrimp, *Lysmata vittata* as an effective biocontrol agent under simulated recirculating marine aquaculture conditions, and its potential for use on fish farms. The ability of *L. vittata* to consume *N. girellae* eggs on cage netting is significant because the eggs constitute the main source of reinfection to fishes in aquaculture (Shirakashi and Hirano 2015), and are resistant to chemical treatments used to control adult parasites on the host fishes (Whittington and Kearn 2011). The traditional method to control *N. melleni* egg accumulation on farms is the manual replacement of nets, however, the most efficient timing of net changes is unknown, and the daily accumulation of *N. girellae* eggs exacerbates an already labour intensive and time consuming farm practice (Shirakashi and Hirano 2015). Recently, Shirakashi and Hirano (2015) evaluated some of the distribution dynamics of *N. girellae* eggs in a culture cage in support of the development of novel future egg removal methods. I believe that *L. vittata*, and possibly other shrimp species may offer this novel solution, particularly for land-based operations, broodstock facilities, hatcheries and nurseries because shrimp are capable of locating and consuming these eggs, which offer a rich source of protein and lipids, including saturated, monounsaturated, and polyunsaturated fatty acids (Brazenor *et al.* 2017b).

To the best of my knowledge, the data also demonstrate for the first time the sudden increase in intensity, and the intensity range of *N. girellae* post-larvae for an entire susceptible captive host population within days of an initial benign infection. A sudden outbreak of *N. girellae* (*sec*. Brazenor *et al.* 2018) was considered the reason for the acute mortality of 200 000 farmed barramundi, *Lates calcarifer* in the Hinchinbrook Channel in Northern

Queensland, Australia (Deveney *et al.* 2001). A contributing factor was thought to be a precluding period of unfavourable environmental conditions for the fish. Following this initial mass mortality event, and the return to optimal environmental conditions, the surviving fish appeared to make a rapid recovery (Deveney *et al.* 2001).

After the initial sudden, high-intensity infection in the results, I observed an overall decrease in the number of parasites on fish in both treatment groups over time by approximately 37%, independent of the shrimp treatment. This reduction in numbers did not influence the significant effect of *L. vittata*, and is thought to be a natural reduction in parasite intensity through competition as the parasites grow and seek out specific sites on the host skin (see Trujillo-González *et al.* 2015), combined with the removal of some individuals from host flashing in response to infection (Shirakashi and Hirano 2015), and the function of the host immune response (Bondad-Reantaso *et al.* 1995). Host mortality is directly proportional to parasite intensity (Grenfell and Dobson 1995) and infection duration (Fox *et al.* 2018). Prior to the experiment, the host fish used were naïve to *N. girellae* infection. My data and the investigation of Deveney *et al.* (2001) highlight the importance of the role of host immunity in outbreaks of *N. girellae* since the unfavourable environmental conditions in the Hinchinbrook Channel example likely contributed to temporary immune-suppression (Deveney *et al.* 2001), and my fish had no prior acquired immunity. In this regard, *L. vittata* not only reduced infection pressure, but also the intensity range of *N. girellae*, possibly reducing the risk of potential mortality.

Cleaner shrimp in both tropical and temperate environments are known to prey on the ectoparasites of fishes (see Vaughan *et al.* 2016; Chapter 2 for species), but it is likely that only the gregarious species, like *L. vittata*, would offer any meaningful benefit to aquaculture, as individuals of these species naturally occur together in groups, unlike the pair-forming species which are intolerant of additional conspecifics (Wong and Michiels 2011). *Lysmata vittata* has

a natural distribution extending throughout the Indo-Pacific (Palomares and Pauly 2018), which includes the major marine aquaculture producing nations. It is currently cultured commercially in Australia for the ornamental trade, and has the potential for large scale development. *Lysmata vittata* may also be effective against other fish parasites in aquaculture. In my recent laboratory trials, *L. vittata* was effective at reducing and consuming the benthic stages of the ciliophoran ectoparasite *C. irritans*, and the cocoons of the marine leech *Z. arugamensis* (Chapter 4). Its efficiency against these and other parasites remains to be tested under farm conditions. However, cleaner shrimp biocontrol models may offer a solution to sympatric infestations, which are often the reality in aquaculture. Additionally, cleaner shrimp may also reduce the invasion of injuries by secondary pathogens by promoting wound healing (Chapter 3). Cleaner shrimp and other shrimp species should therefore continue to be explored for a future role in aquaculture biocontrol, as originally proposed by Becker and Grutter (2004). Hints of the success of using shrimp in biocontrol already exist in the literature; *R. typus* has been used to reduce net biofouling of scallop cages in Chile (Dumont *et al.* 2009), while the experimental field trial use of the native freshwater shrimp *Macrobrachium vollenhoveni* (Herklots, 1857) to parts of the Senegal River, reduced the prevalence of human schistosomiasis by predation on the snail intermediate host (Sokolow *et al.* 2015).

Historically, the cleaner fishes' biocontrol model has contributed significantly to the reduction of sea lice in European salmon farming (González and de Boer 2017), and reduced the reliance on drugs and chemical treatments to control sea lice outbreaks (Treasurer 2002; Powell *et al*. 2017), thereby reducing the impact of disease, and mandatory drug withdrawal periods prior to harvesting. The development and application of cleaner shrimp biocontrols could have a similar result in aquaculture, particularly in sub-tropical and tropical regions, where stock losses from ectoparasites are high (Shinn *et al*. 2015), and where cleaner fishes are an unlikely option. The global financial loss from pathogens in aquaculture is estimated to

be approximately 20% of total production value (Sitjá-Bobadilla and Oidtmann 2017). Financial losses are linked to livestock mortalities, the impact of non-lethal infections on livestock growth performance, the market rejection of diseased livestock (e.g. Ogawa 1994; Moran *et al.* 1999), and the associated costs of mitigating diseases (Lafferty *et al*. 2015). Diseases in general are considered the most significant constraint to future global aquaculture expansion (Stentiford *et al*. 2017), and will undoubtedly be influenced by the increasing incidence of pathogen resistance to treatments (*cf.* Conly and Johnston 2005; Done *et al*. 2015; Watts *et al.* 2017). Indeed, the development of the cleaner fishes' model was driven largely by the increase in resistance of sea lice to chemical therapies (Costello *et al.* 2001; Costello 2006; Aaen *et al.* 2015). It is therefore likely that alternative controls against ectoparasites in finfish aquaculture will continue to attract increasing interest and support globally. Biocontrols offer considerable potential in this regard, particularly if included as part of a holistic integrated pest management strategy (Mordue and Pike 2002; Brooks 2009; Sitjá-Bobadilla and Oidtmann 2017) to combine multiple dynamic approaches to disease challenges (Aaen *et al.* 2015).

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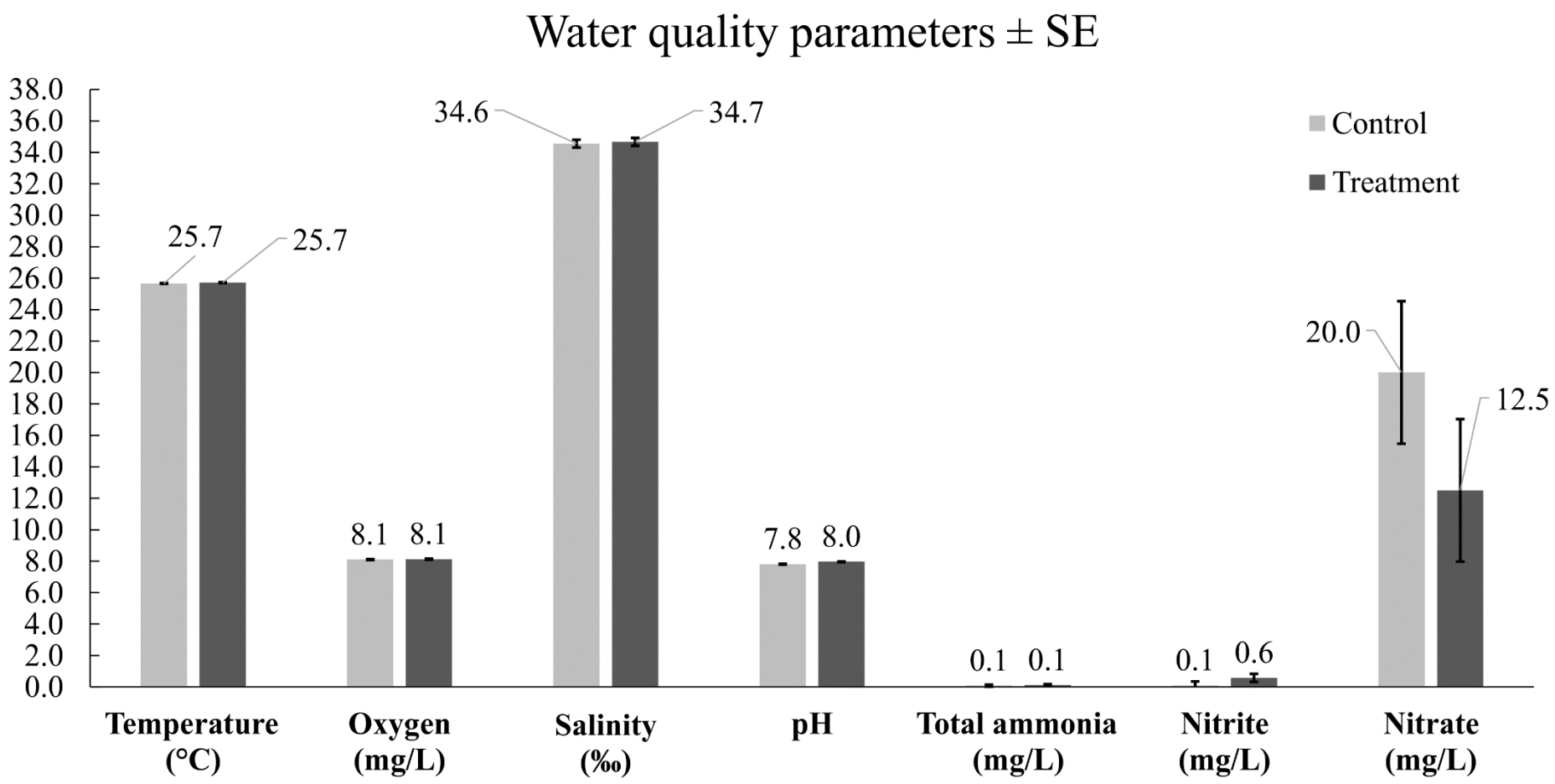

Fig. 5.1. Water quality parameters recorded for the duration of the experiment.

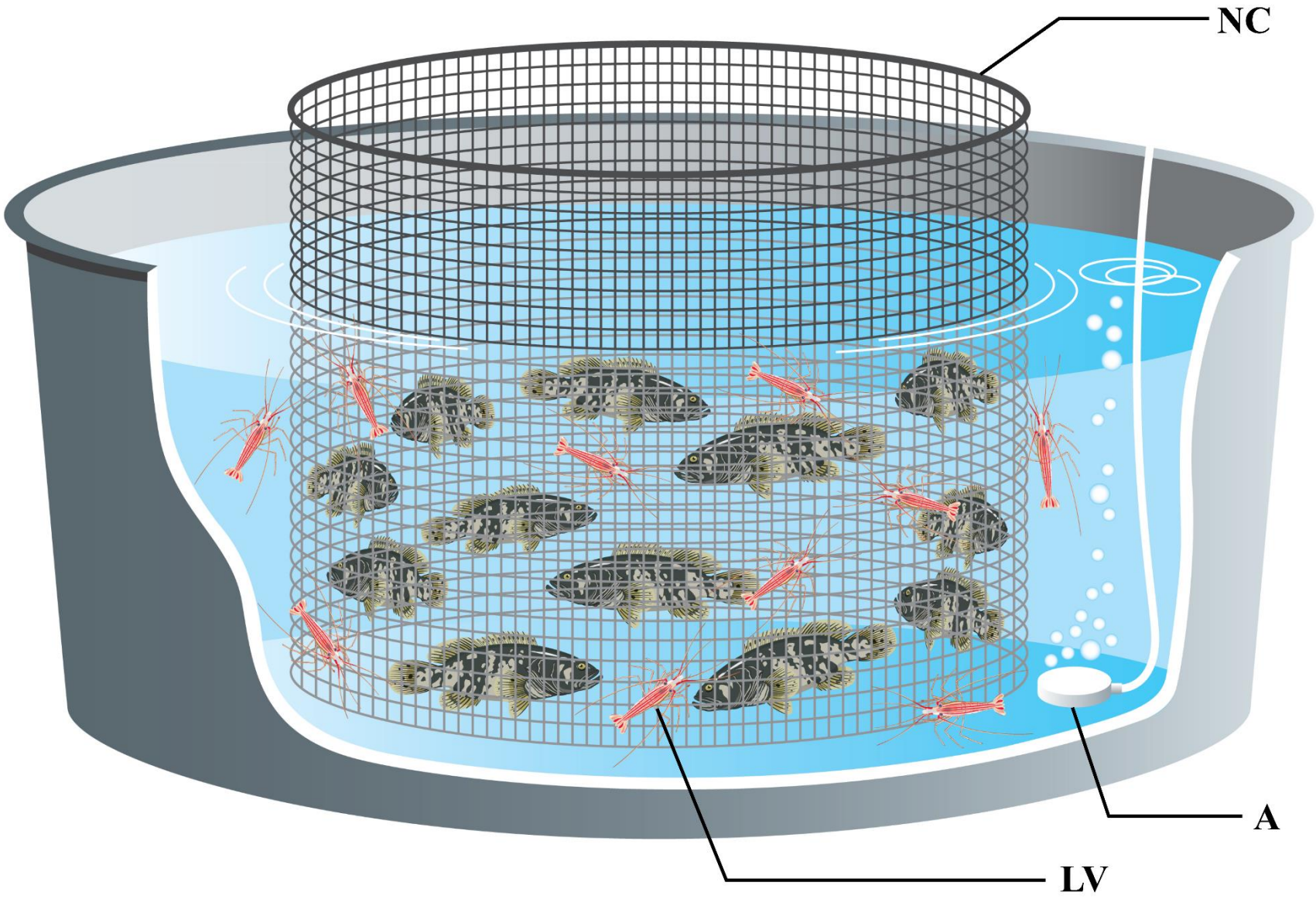

Fig. 5.2. Graphic representation of a replicate treatment tank containing juvenile *Epinephelus lanceolatus* inside an oyster mesh net cage (NC), and *Lysmata vittata* (LV) on the outside of the cages. A = constant aeration.

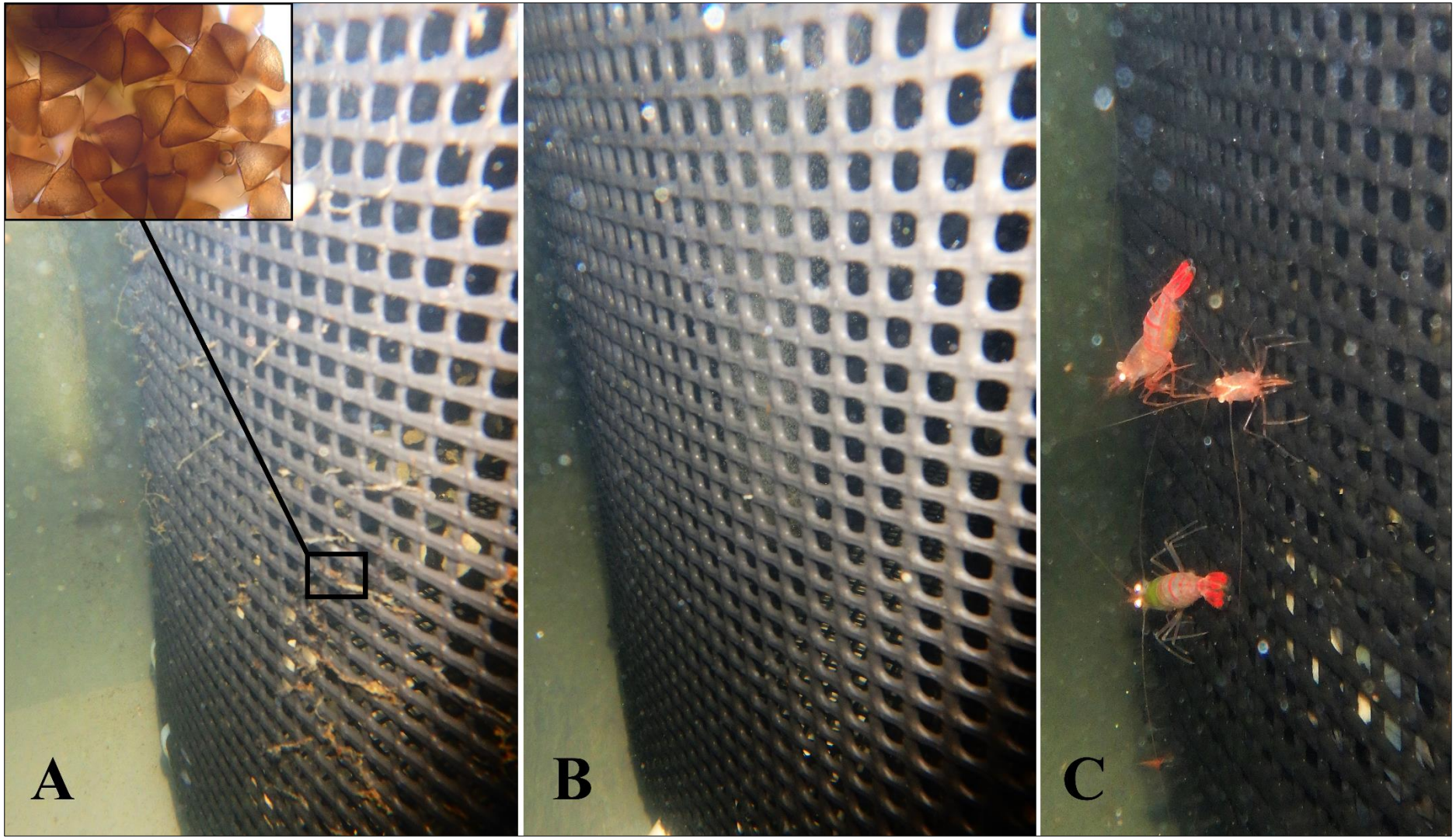

Fig. 5.3. Underwater photographs of net cages during experimentation; A. control cage (absence of *L. vittata*) with accumulation of *Neobenedenia girellae* egg masses; B. treatment cage (presence of *L. vittata*); C. *Lysmata vittata* patrolling on the external surface of a net cage.

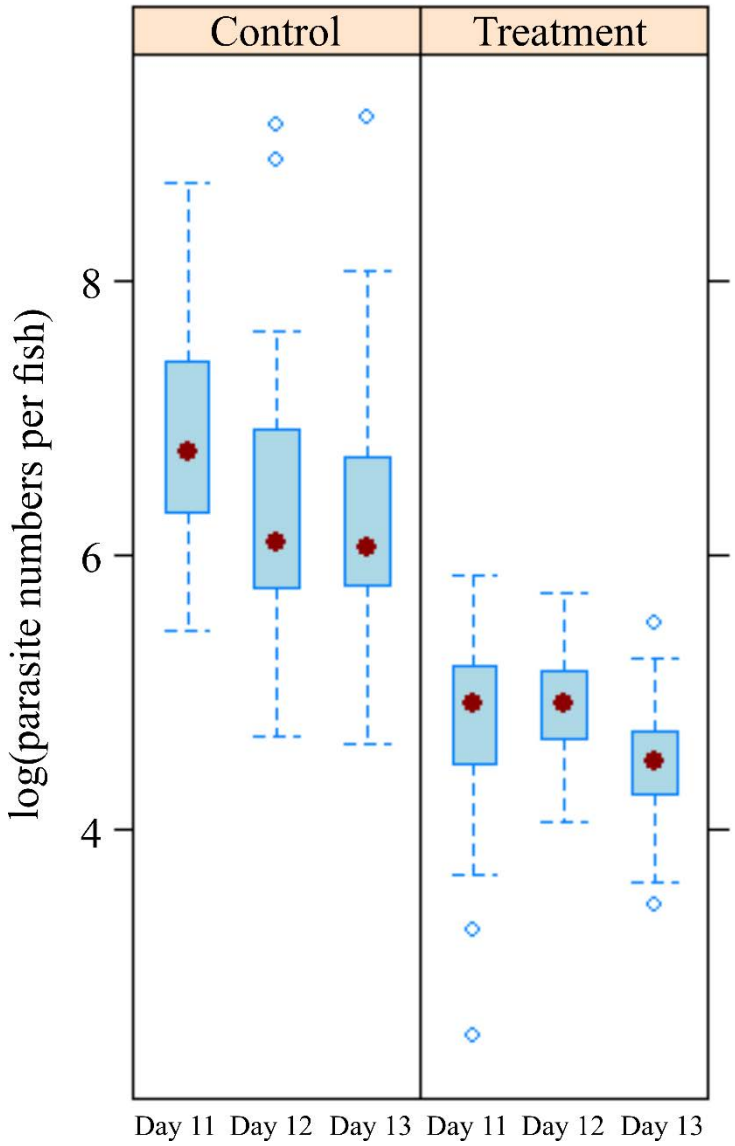

Fig. 5.4. The effect of *Lysmata vittata* on the number of *Neobenedenia girellae* infecting juvenile *Epinephelus lanceolatus*. Outliers generated by the analysis are represented as clear circles. Day = day post-infection.

Table 5.1. Fixed effects regression results

Fixed effects	**β**	**95% confidence intervals**
Intercept	6.88	[6.65, 7.10]
Treatment (shrimp)	-2.11	[-2.50, -1.73]
Day (Day2)	-0.62	[-0.86, -0.37]
Day (Day3)	-0.60	[-0.83, -0.38]
Treatment (shrimp): Day (Day2)	0.75	[0.47, 1.03]
Treatment (shrimp): Day (Day3)	0.32	[0.06, 0.60]

CHAPTER 6

General Discussion

Cleaning symbiosis knowledge over the last half century attempted to clarify the representative cooperative, mutualistic, interspecific relationships between animals based on the benefits both parties receive. These benefits, specific to cleaning symbioses, included a food resource for the cleaner, and the reduction of parasites, other pathogens, epibionts, and dead or diseased tissues directly off the client (e.g. Feder 1966). However, it was necessary to re-evaluate the cleaning symbiosis concept and to amend its definition to highlight the fundamental importance of communicative behaviour as a predisposing catalyst for all cleaning symbiotic interactions (see Vaughan *et al*. 2016; Chapter 2). Without this distinction, the concept of cleaning symbiosis was confused with a related but separate mutualism, 'incidental cleaning', which, unlike cleaning symbiosis, required no specific behavioural or morphological evolutionary adaptations of either client or cleaner (Côté 2000; Vaughan *et al*. 2016; Chapter 2).

The separation of the two mutualisms, and the re-evaluation of cleaning symbiosis, has been met with general acceptance (Artim *et al.* 2017; Dunkley *et al.* 2017; González and de Boer 2017; Grutter *et al*. 2017; Soares 2017; Titus *et al*. 2017; Quimbayo *et al*. 2017; Binning *et al.* 2018; Bos and Fransen 2018; Horká *et al.* 2018; Moura *et al.* 2018; Sikkel and Smit 2018; Quimbayo and Zapata 2018; Quimbayo *et al.* 2018). This re-evaluation allowed for a stock-take of global aquatic cleaner organisms (see Vaughan *et al.* 2016; Chapter 2, Tables 2.1, 2.2), addressing the first aim of this thesis, and has recently also provided support for the development of recent evolutionary hypotheses in fish-cleaning cleaner shrimp (Horká *et al.* 2018).

Cleaner shrimp cleaning abilities were the focus of this thesis because, until recently, these shrimp remained underinvestigated by the research community in favour of more

charismatic cleaner fish species. Only four empirical studies had been published on the specifics of fish ectoparasite removal by cleaner shrimp under laboratory conditions, and one from a semi-natural macrocosm, over the last 20 years (Vaughan *et al.* 2016; Table 1.1). Prior to the work presented in this thesis, it remained unknown whether cleaner shrimp tended honestly, through truly symbiotic interactions, to injured clients. Previous historic literary accounts speculated that they may (Limbaugh 1961; Limbaugh *et al*. 1961; Corredor 1978; Crump 2009), but provided no supporting evidence. It remained unknown whether any of these purported interactions were not merely suggestions, or simply observed *in situ* examples of opportunistic parasitism on compromised clients.

The results of Chapter 3, in fulfilment of the second thesis aim, represented the first evidence of true symbiotic cleaning of injured client fish by cleaner shrimp. This further confirmed the importance of communication in cleaning symbioses, and highlighted the equal importance of cleaner shrimp to cleaner fishes in maintaining the health of reef fishes. Thus, cleaner shrimp have the ability to regulate fish ectoparasite populations on the reef. This chapter also provided further insight into the benefits of cleaner shrimp in client wound management, demonstrating for the first time a direct influence of cleaning by the shrimp on the client fish's inflammatory process, which has never been demonstrated before for any cleaner organism. The lack of comparable influences of other cleaner organisms on client fish injury-associated inflammation is not an indication of cleaner shrimp superiority, rather, it represents a lack of research in general on the influence of other cleaning organisms on client injuries.

Currently, mechanism underlying the reduction of inflammation by the shrimp remains unresolved, but the ecological significance of its consequence for client fishes is likely considerable (*cf*. Barber *et al.* 2000). For example, recent research suggested that injured fish [in the absence of cleaners] were more likely to be parasitised by ectoparasites than their

uninjured counterparts (Jenkins *et al*. 2018), which could be a function of stress (see Bshary *et al.* 2007). Injuries are also known to be vulnerable to invasion by secondary infections by opportunistic bacteria and viruses (Fontenot *et al.* 2004; Jensen *et al.* 2015; see also Appendix 5; Table A5.1). However, the reduction of inflammation, and therefore the reduction of increased levels of blood products at the site of injury, may influence the ability of some pathogens to colonise. The cleaning of injuries by cleaner organisms may therefore reduce or remove this increased likelihood of parasitism of injured fishes observed by Jenkins *et al.* (2018), and may also reduce the colonisation by common secondary opportunistic pathogens. In addition, the mere presence of cleaners, including cleaner shrimp is known to have a mitigating effect on client stress (see Bshary *et al*. 2007). These factors would be expected to support the return of an injured fish's behaviour to a degree of normality as the injury healed (see Foster 1985; Barber *et al.* 2000).

The mechanism of rapid wound sealing by a uniform layer of migrating epithelium is well documented in fishes (e.g. Fontenot and Neiffer 2004; Guerra *et al.* 2008; Böckelmann *et al.* 2010), and is crucial to restore homeostasis (Bereiter-Hahn 1986). Cheating by the cleaner shrimp in Chapter 3 was assumed to involve evidence of damage to this delicate monolayer of cells, thus disrupting the healing process. Minor damage to the epithelium of the body margin of client fish was detected in the analyses, but was not associated with shrimp, rather, it was indicative of handling (Chapter 3, Fig 3.7).

The improvement in the methodology to quantify tissue damage from photography (Chapter 3) may offer a useful tool for further investigating cheating in cleaning symbiosis, as the above technique is highly sensitive for epithelial damage, which may be more directly influenced by cheating cleaner fishes, rather than shrimp. Currently however, cheating by cleaners, including cleaner shrimp, is measured by proxy, recorded as client flinches or jolts as a presumed response to cleaners removing clients' skin, scales or mucus (e.g. Bshary and

Grutter 2002; Whiteman and Côté 2002; Soares *et al.* 2008b; Oates *et al.* 2010; Titus *et al.* 2017). The results of Chapter 3, specifically those of the analysis of jolt-rate (Fig. 3.4), suggest that client idiosyncrasy is a previously neglected consideration of cheating behaviour, and therefore that a more direct measure of tissue damage associated with cheating may be more accurate in future.

Cleaner shrimp idiosyncrasy did not have an effect on the jolt-rate model (see Appendix 2), but different cleaner shrimp species were shown to perform differently against the removal of different ectoparasites on fish, and their environmental stages (see Chapter 4). Previous research on the ability of cleaner shrimp to remove fish ectoparasites (Table 1.1) reflected the pioneering studies to verify the outcomes of original, incidental observations (e.g. Östlund-Nilsson *et al.* 2005), or single ectoparasite models to add support to the hypothesis that "cleaner shrimp do clean" (Becker and Grutter 2004), or to evaluate shrimp effect on ectoparasite size and abundance (e.g. McCammon *et al.* 2010). No research had specifically attempted to evaluate the performance of different cleaner shrimp species against different parasite models, and therefore to begin to consider their potentially different ecological functions. Prior to the work presented in this thesis, cleaner shrimp were only recorded to remove and to consume either crustacean or platyhelminth parasite taxa (see Table 2.3). However, protists and hirudinean ectoparasites are common on reef fishes, and are problematic groups in ornamental and finfish aquaculture (Shinn *et al.* 2015).

In Chapter 4, I fulfilled the third research aim by using a combined approach to add to the understanding of cleaner shrimp behaviour and cleaning abilities. I evaluated four cleaner shrimp species' diel ectoparasite reduction performance against three different ectoparasite models representing the phyla Annelida, Ciliophora, and Platyhelminthes, while targeting the results specifically to a need to explore novel biocontrols in marine finfish aquaculture. The results of Chapter 4 demonstrated that different cleaner shrimp on the reef likely offer different

levels of service quality to clients, possibly in relation to the types of parasites they harbour, which supports the earlier suggestion of a cleaner discordance between cleaner organisms by Titus *et al.* (2015). Whether clients, which may host species-specific ectoparasites or epibionts select the best cleaner shrimp species based on different service performance, remains unknown, and should be investigated further. Previous work has eluded to this possibility.

Sazima *et al.* (2004a) reported Hawksbill turtles (*Eretmochelys imbricata* Linnaeus, 1766) repeatedly visited *S. hispidus* to have their epibionts removed during the day. Members of the genus *Stenopus* are unique morphologically, and have the largest chelae of any of the cleaner shrimp, reminiscent of lobsters. This morphology may predispose them to a specific role in removing larger parasites or epibionts from clients, such as leeches. Until recently, the ability of *S. hispidus* as a remover of fish ectoparasites remained apparently uncertain, citing poor performance against smaller ectoparasites like *Neobenedenia* sp. (e.g. McCammon *et al.* 2010). Certainly, *S. hispidus* did perform poorly against *N. girellae* in the parasite trials of Chapter 4, but was highly effective at removing larger leeches (*Z. arugamensis*) off client fish. In a further investigation (see Appendix 6), *S. hispidus* was the only shrimp species tested that reflected long-wave ultraviolet from specific markings on its body, and interestingly, Hawksbill and other marine turtles are visually sensitive to these wavelength (Fritsches and Warrant 2013; Wang *et al.* 2013), which would also only have a diurnal function.

Urocaridella antonbruuunii was recently photographed by Bos and Fransen (2018) cleaning *Siganus canaliculatus* (Park, 1979) at night, adding to the suggestion of Bonaldo *et al.* (2014) that some cleaner shrimp function nocturnally. Bos and Fransen (2018) suggested that *U. antonbruunii* may be a specialised nocturnal cleaner. However, this species demonstrated equal diurnal and nocturnal cleaning ability of fish infested with *N. girellae* in the trials presented in Chapter 4, and the general results indicated that nocturnal cleaning may be different between cleaner shrimp species, or specific to the ectoparasitic infection that the

clients host. For example, a pattern was apparent in the results of experimentation with the ciliate *C. irritans* on infected fish. None of the shrimp reduced the parasitic stage on the fish during the day, while all shrimp reduced their numbers at night, albeit with different degrees of success (Figs 4.2, 4.6). Whether the nocturnal activity of *C. irritans* on the host fish, or the behaviour of the host fish itself renders the ciliates more susceptible to predation by cleaner shrimp at night remains unknown. What is evident though, is that cleaning by shrimp is dynamic, and may be influenced by several variables, some of which are yet to be identified. Certainly, both dedicated and facultative cleaner shrimp (e.g. *L. amboinensis*, and *L. vittata*, respectively) can be highly efficient at ectoparasite benthic stage reduction in the environment and may therefore present an additional benefit to clients by reducing ectoparasite reinfection pressure. This benefit is of particular interest when considering cleaner shrimp as potential biocontrols.

Future work might consider involving multiple infections models, which is reflective of a more likely scenario in aquaculture. It is unknown how shrimp might respond to a multiple species infestation on the same client; whether they might have a preference for a single parasite species or a preference for size. However, a notable challenge here for exploration of multiple species infestations under controlled laboratory conditions is the potential impact on client fish welfare. Under current ethics regulations it would be unlikely that such models would be considered ethical.

Chapter 5 represented the first ever exploration of a candidate cleaner shrimp as a biocontrol under aquaculture conditions, demonstrating the value of the investigation of this novel approach. The only previous consideration of using cleaner shrimp as potential biocontrols against fish ectoparasites in aquaculture was made by Militz and Hutson (2015), after the original suggestion of Becker and Grutter (2004). The work of Militz and Hutson (2015) demonstrated the effect of *L. amboinensis* against *N. girellae* eggs and the invasion

success of the infective larvae against individual ornamental sea goldies, *Pseudanthias squamipinnis* (Peters, 1855). The monogenean *N. girellae* is a serious problem in finfish aquaculture throughout the Asia-Pacific region (Shinn *et al*. 2015), but it remained unknown whether *L. amboinensis* and other cleaner shrimp species would be effective against other economically important fish ectoparasites, and whether any cleaner shrimp could be used effectively as a biocontrol under aquaculture conditions. It was evident that the performance of a potential candidate cleaner shrimp biocontrol agent for fish ectoparasites in aquaculture had to demonstrate a high degree of efficacy across the diversity of problematic ectoparasite taxa, and would need to be flexible to the limitations imposed by the aquaculture conditions.

Lysmata vittata was identified as the ideal candidate species from its superior performance specifically against the benthic life-stages of the different ectoparasites tested in Chapter 4. These stages are traditionally the main source of repeated reinfections in aquaculture (e.g. Shirakashi and Hirano 2015), and are generally resistant to chemical therapies employed to treat the parasitic stages on fishes (e.g. Whittington and Kearn 2011). *Lysmata vittata* presented an advantage over both *L. amboinensis* and *S. hispidus* in that it is a widely distributed gregarious species and therefore it can be used in large numbers. Both *L. amboinensis* and *S. hispidus* are territorial and pair-forming, and can be aggressive to other members of the same species (Wong and Michiels 2011).

Under simulated aquaculture conditions, *L. vittata* successfully reduced reinfection pressure of *N. girellae* by consuming the eggs attached to the cages housing fish (see Chapter 5). This successful reduction of *N. girellae* numbers by *L. vittata* carries considerable implications for marine finfish aquaculture. The targeting of benthic life-stages of fish ectoparasites that are traditionally responsible for multiple reinfections, is uniquely offered by cleaners shrimp, and suggests a reduction in the labour costs and time spent replacing or disinfecting contaminated infrastructure. However, the superior benefit to farmers would be

the reduction of the impact that infectious ectoparasites impose on cultured stock health, and the subsequent improvement of the overall yield. Equally important in aquaculture is the need to find ways to reduce the use of chemical and drug therapies to reduce the risk of the development of drug or chemical resistance by various pathogens (Aaen *et al.* 2015), and the environmental impacts that may be associated with their use (Langford *et al.* 2014). An improvement in the management of problematic ectoparasites by employing cleaner shrimp as biocontrols in regions such as the Asia-Pacific, could reduce the reliance on traditional chemical treatment interventions and drug therapies, especially metaphylaxes, as was the case in Europe with the introduction of cleaner fishes in Atlantic salmon farming (Treasurer 2002; Aaen *et al*. 2015; Powell *et al.* 2017).

Repetitive use of chemical therapies and drugs, their use off-label without consideration for an informed treatment strategy, and the use of agents with a persistent half-life, all support an increased risk of ectoparasites and other pathogens in aquaculture developing resistance (Aaen *et al*. 2015). The mechanisms of drug and chemical resistance for many ectoparasites are not fully understood for the diversity of problematic taxa, and have historically only been researched based on the identification and confirmation of resistance as a result of well implemented legislated surveillance programmes (*cf*. Aaen *et al.* 2015; Shinn *et al.* 2015; Watts *et al.* 2017). The reality however remains that in the absence of adequate surveillance, training, and legislation in over 90% of global aquaculture countries (Watts *et al*. 2017), the incidence of pathogen resistance, including ectoparasites in aquaculture is likely to be highly underestimated, or remains currently unknown. Therefore, the application of biocontrols in future, such as *L. vittata* in aquaculture, is in my opinion likely to become a necessity as other more traditional management options become increasingly ineffective, rather than being considered a novelty. Indeed, one only need consider the reasons for the development of the

salmonid cleaner fish model in retrospect, to offer a glimpse into the future use of cleaner shrimp and other biocontrols in global aquaculture.

Diseases in aquaculture represent a major developmental impediment (Stentiford *et al.* 2012), yet production is required to double by 2050 to meet the global demand (Stentiford *et al.* 2017). As aquaculture expands globally, increased risk of diseases, and disease emergence is inevitable. Currently there are no commercial vaccines available against fish ectoparasites (Sommerset *et al*. 2005; Dadar *et al.* 2017), and therefore aquaculture farmers faced with ectoparasite problems have few alternatives to chemical and drug treatments. Biocontrols, such as the use of cleaner shrimp like *L. vittata* may offer a more sustainable solution, and if managed correctly as part of an integrated strategy in aquaculture (Sitjá-Bobadilla and Oidtmann 2017), may deliver multiple benefits. However, before assigning merits of panacea to biocontrols in aquaculture, it is important to understand their limitations, and to realise that novel solutions often present their own novel problems. This is certainly true for cleaner fish used in salmon aquaculture, which have not been immune to reports of disease transmission problems and environmental concerns (see Karlsbakk *et al*. 2013, 2014; Karlsbakk 2015; Haugland *et al.* 2017; Powell *et al*. 2017; Faust *et al.* 2018). Therefore, an important consideration of using cleaner shrimp in aquaculture would be to initially investigate the potential risks they may pose themselves for broader pathogen transmission, and any impact they might pose to the environment, before being generally implemented. In addition, applications of cleaner shrimp as biocontrols must also consider their potential sensitivity to concurrent applications of chemical parasiticides. Certainly there are chemicals specifically designed to kill crustacean parasites which may likely have a negative impact on any concurrent applications of shrimp biocontrols. In this regard however, a balance and treatment application planning (see discussion points by Sitjá-Bobadilla and Oidtmann 2017) are fundamentally important for the success of shrimp as a biocontrol.

Currently, few species of cleaner shrimp have been domesticated because of the complexity of the life-cycle of many species, however, the technology to do so is developing (e.g. Hettiarachchi and Edirisinghe 2018). The focus on captive production of cleaner shrimp is for the ornamental trade market, and has not yet been considered for large-scale production to meet the demands of an aquaculture industry. However, improvement in captive production technology, and an additional market demand could foreseeably spark an increased level of interest in cleaner shrimp aquaculture.

Future research directions

The investigation of cleaner shrimp cleaning abilities in this thesis, and the exploration of them as potential biocontrols in aquaculture, raised addition questions which would be valuable to pursue in future. These might include:

1. The further investigation of the mechanism underlying the ability of cleaner shrimp and possibly other cleaner organisms to reduce client injury-related inflammation. This would need to be designed as a more discreet analysis of cellular pathology over the entire period of healing, but an understanding of this mechanism may greatly add to our understanding of the symbiotic cleaning interactions, and the fate of injured fish on a reef.
2. It remains unknown whether there is a co-evolutionary link between clients, their specific parasite fauna, and different cleaner shrimp. A cleaner discordance has been identified previously (see Titus *et al*. 2015), which suggests that different cleaners offer different cleaning services. Different cleaner shrimp morphologies and abilities may predispose them to specific roles or to specific client types which may harbour specific parasites. An *in situ* investigation of

the client range of different cleaner shrimp together with an understanding of the parasite and/or epibiont fauna of these clients may offer a good beginning point.

3. The investigation of the diversity of prey items naturally consumed by different cleaners shrimp in the wild. This could be accomplished by using molecular methods similar to O'Rorke *et al.* (2012, 2014). This information could further support the investigation of the specific functions of different cleaner shrimp species, and could elucidate the diversity of pathogens and parasites that cleaner shrimp do reduce on coral reefs.
4. The development of an efficiency ratio of cleaner shrimp numbers to fish biomass in aquaculture against different economically important ectoparasites. The experimental design employed in Chapter 5 could not accommodate the investigation of this query specifically, yet, a future approach with a larger sample and greater replicates could explore predictive values possibly using negative binomial regression analysis. The results of which would likely be species-specific.
5. The closure of the life-cycle and domestication of different cleaner shrimp species for application in aquaculture. Currently, only *L. vittata* is considered domesticated and is produced commercially in Tasmania. However, after the completion of Chapters 4 and 5, all shrimp from this thesis were donated to further study at James Cook University in Townsville, and researchers here have since successfully bred and raised *Urocaridella antonbruunii*.
6. The susceptibility of *L. vittata* to WSSV is unknown, but could hamper efforts to commercialise production for farm use. This requires further investigation.

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Appendix 1. Animal ethics approval

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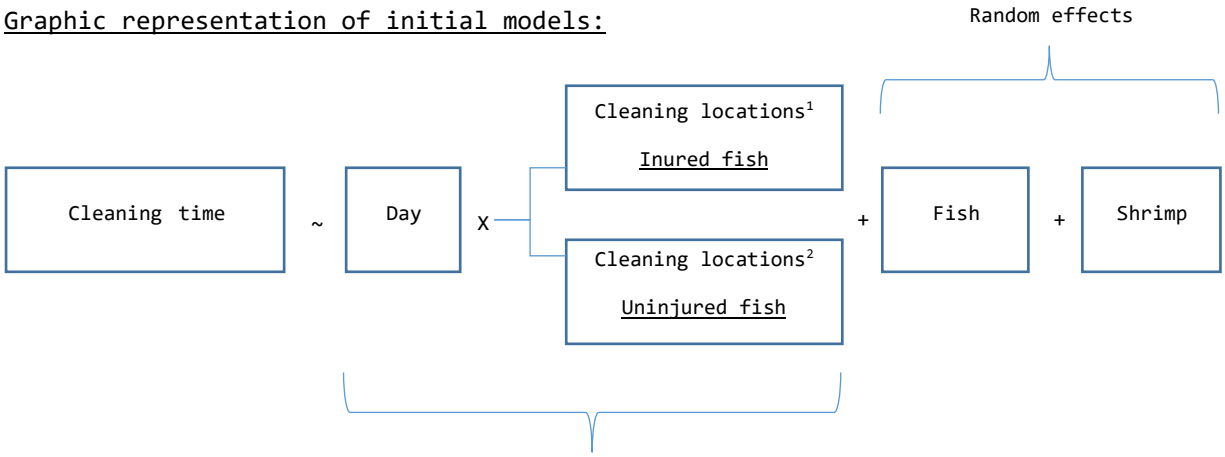
Appendix 2. Details of the statistical analyses for Chapter 3 using *R*

Behavioural analyses

Graphic representation of initial models:

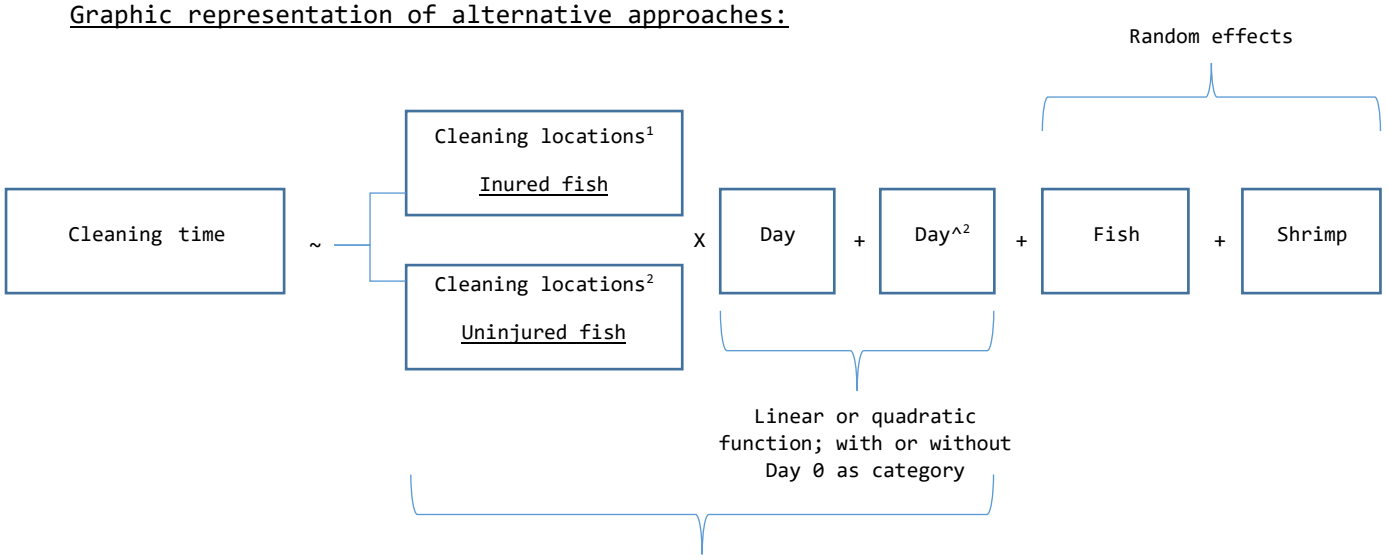


*Cleaning locations¹
- Injured side on injured fish
- Not injured side on injured fish

*Cleaning locations²
- Left side on uninjured fish
- Right side on uninjured fish

And/or Oral and Ventral

Graphic representation of alternative approaches:



*Cleaning locations¹
- Injured side on injured fish
- Not injured side on injured fish

*Cleaning locations²
- Left side on uninjured fish

- Right side on uninjured fish

```
# Behavioural models use packages 'lme4', 'car':
# Sequential tests of fixed effects use a Wald test from the 'car' package
# A: Does cleaning time differ between cleaning locations and over time?
# This initial exploratory model looks at all cleaning locations which
# includes four levels of fish sides (both sides of injured and uninjured
# fish), plus ventral and oral cleaning locations. Subsequent models use a
# data subset excluding oral and ventral cleaning locations to focus on
# comparisons of injured and uninjured sides.  Sections B to D explore the
# most appropriate way to model the effects of time and cleaning location;
# section E shows the final preferred model.

# First model:
>All.lmer = lmer(cleaning_time ~ Day * cleaning_location + (1 | Fish) + (1 |
Shrimp),data=All.data)
> Anova(All.lmer)
Analysis of Deviance Table (Type II Wald chisquare tests)

Response: cleaning_time
                       Chisq Df Pr(>Chisq)
Day                   2.4448  1    0.11791
cleaning_location     377.9274  5    < 2e-16 ***
Day: cleaning_location  9.9174  5    0.07761 .
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1



# B: Does cleaning time or temporal changes in cleaning time,
# differ between injured and uninjured sides?
# Second model excluding oral and ventral interaction locations:

>Second.lmer = lmer(cleaning_time ~ Day * cleaning_location + (1 | Fish) + (1 |
Shrimp),data=All.dataR.14)
> Anova(Second.lmer)
Analysis of Deviance Table (Type II Wald chisquare tests)

Response: cleaning_time
                     Chisq Df Pr(>Chisq)
Day                  3.4968  1    0.06149 .
cleaning_location    1.9222  3    0.58871
Day:cleaning_location 8.4222  3    0.03805 *
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1


# C Does allowing curvature better describe the effect of Day?
# Third model, Day included with both linear and quadratic terms:

> Model3.lmer = lmer(cleaning_time ~ cleaning_location * (Day + I(Day^2)) + (1 |
Fish) + (1 | Shrimp),data=All.dataR.14)
> Anova(Model3.lmer)
Analysis of Deviance Table (Type II Wald chisquare tests)

Response: cleaning_time
                         Chisq Df Pr(>Chisq)
cleaning_location       1.9289  3    0.58729
Day                     6.2952  1    0.01211 *
I(Day^2)                3.9331  1    0.04734 *
```

```
cleaning_location:Day      6.1754  3    0.10338
cleaning_location:I(Day^2) 4.0167  3    0.25966
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

 # D. Does the day effect extend beyond the day of initial injury?
Fourth model, Day 0 excluded
Shows no effect of day or cleaning location with Day 0 removed:

```
> Model4.lmer = lmer(cleaning_time ~ cleaning_location * (Day + I(Day^2)) + (1 |
Fish) + (1 | Shrimp),data=All.dataR.14, Subset: Day > 0)
> Anova(Model4.lmer)
Analysis of Deviance Table (Type II Wald chisquare tests)

Response: cleaning_time
                          Chisq Df Pr(>Chisq)
cleaning_location         2.6754  3     0.4444
Day                       0.9118  1     0.3396
I(Day^2)                  0.8790  1     0.3485
cleaning_location:Day     0.4673  3     0.9260
cleaning_location:I(Day^2) 0.3612  3     0.9481
```

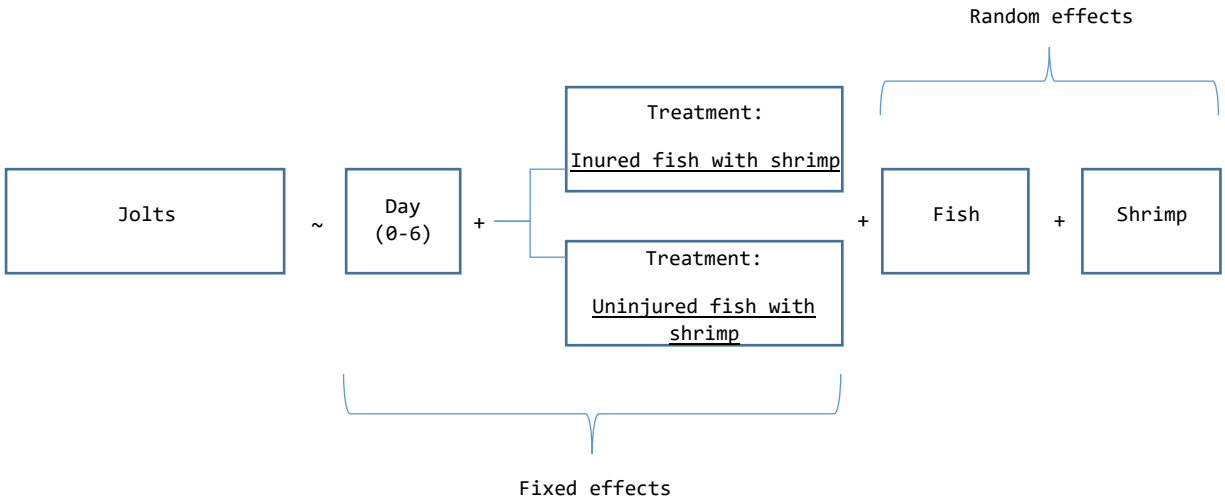
 # E. Final analysis
Fifth model, Day as a binary categoric variable
(isDay0 = True or False),
confirms Day 0 effect varies between cleaning locations

```
> Model5.lmer = lmer(cleaning_time ~ cleaning_location * isDay0 + (1 | Subject) +
(1 | Shrimp),data=All.dataR.14)
> Anova(Model5.lmer)
Analysis of Deviance Table (Type II Wald chisquare tests)
Response: cleaning_time
                        Chisq Df Pr(>Chisq)
cleaning_location       1.9794  3   0.576691
isDay0                  7.5341  1   0.006054 **
cleaning_location:isDay0 9.3244  3   0.025275 *


> confint(Model5.lmer)
Computing profile confidence intervals ...
                                    2.5 %      97.5 %
.sig01                          0.19213809  0.34210407
.sig02                          0.12235332  0.26865399
.sigma                          0.41993828  0.48678741
(Intercept)                     1.85481519  2.16861666
cleaning_location 2            -0.23101041  0.03660577
cleaning_location 3            -0.21046707  0.18919327
cleaning_location 4            -0.25772792  0.14226903
isDay0TRUE                     -0.71797544 -0.23326499
cleaning_location 2:isDay0TRUE -0.05944865  0.60981872
cleaning_location 3:isDay0TRUE  0.13740865  0.82006798
cleaning_location 4:isDay0TRUE  0.11228058  0.77086096
```

The above provides Table 3.1 in the text

Jolting

Graphic representation of jolting model:




```
## Analysis of jolting

# Is jolting related to cheating, or is it fish idiosyncrasy?
# Sequential tests of fixed effects use a Wald test (package 'car')

> Jolts.lmer = lmer(Jolts ~ DayJ + TreatmentJ + (1 | FishJ) + (1 |
ShrimpJ),data=Jolts.data1)
> Anova(Jolts.lmer)
Analysis of Deviance Table (Type II Wald chisquare tests)

Response: Jolts
            Chisq Df Pr(>Chisq)
DayJ       4.3979  6     0.6230
TreatmentJ 1.4340  1     0.2311

# Test for idiosyncrasy of shrimp on the model, compare models with and without
shrimp as a random effect:

> Jolts_model1=lmer(Jolts~DayJ+TreatmentJ+(1|FishJ)+(1|ShrimpJ),data=Jolts.data1)
> Jolts_model2=lmer(Jolts~DayJ+TreatmentJ+(1|FishJ),data=Jolts.data1)
>
> anova(Jolts_model1,Jolts_model2)
refitting model(s) with ML (instead of REML)
Data: Jolts.data1
Models:
Jolts_model2: Jolts ~ DayJ + TreatmentJ + (1 | FishJ)
Jolts_model1: Jolts ~ DayJ + TreatmentJ + (1 | FishJ) + (1 | ShrimpJ)
             Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
Jolts_model2 10 986.25 1022.2 -483.12   966.25
Jolts_model1 11 987.35 1026.9 -482.67   965.35 0.9012      1     0.3425

## Shrimp had no effect on the model as a random effect
```

```
# Test for idiosyncrasy of Fish on the model, compare models with and without Fish
as a random effect:

> anova(Jolts_model1,Jolts_model2)
refitting model(s) with ML (instead of REML)
Data: Jolts.data1
Models:
Jolts_model2: Jolts ~ DayJ + TreatmentJ + (1 | ShrimpJ)
Jolts_model1: Jolts ~ DayJ + TreatmentJ + (1 | FishJ) + (1 | ShrimpJ)
             Df     AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
Jolts_model2 10 1041.74 1077.7 -510.87  1021.74
Jolts_model1 11  987.35 1026.9 -482.67   965.35 56.391      1  5.939e-14 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

## Fish had a significant effect on the model as a random effect
```

White light (colour) analyses

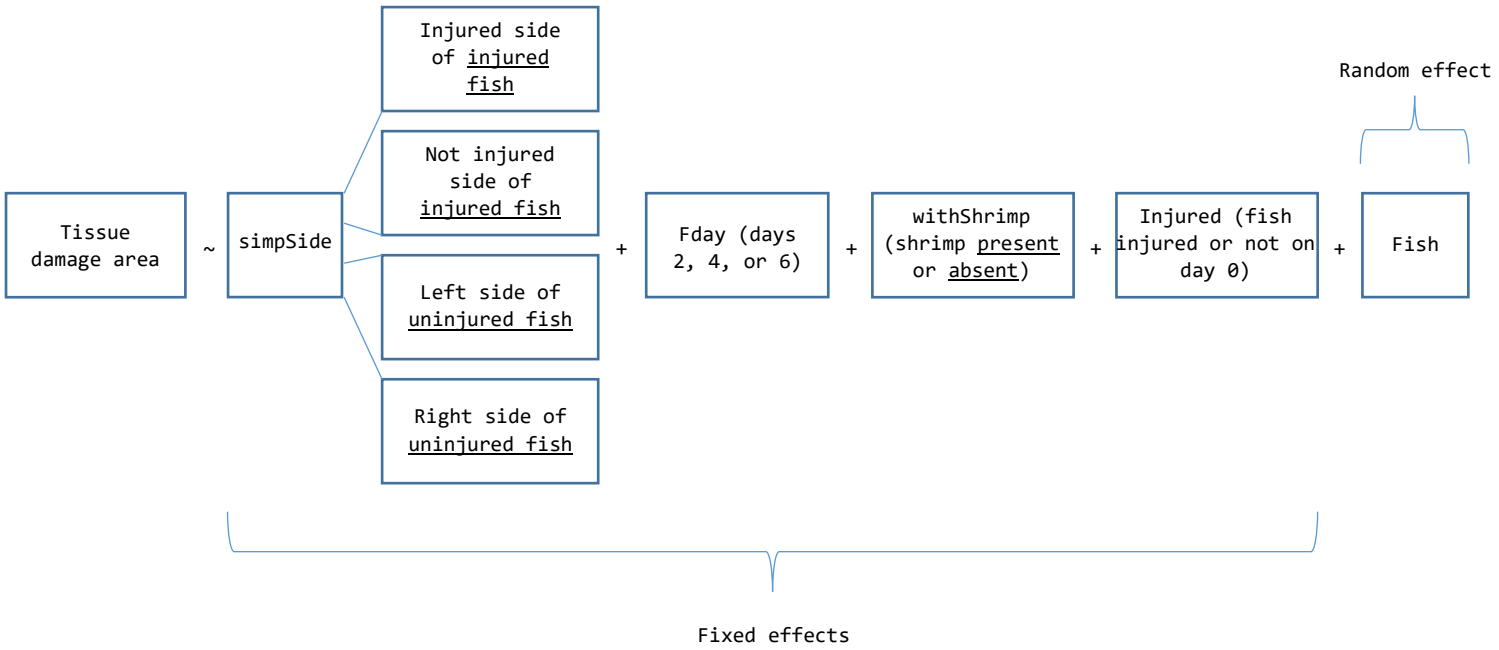
```
## Analysis of redness at injury site (the a-channel for white light)
## IWS – Injury with shrimp; INS – Injury without shrimp

# A:  Is there an increase in redness at the injury site
# immediately post-injury(between day 0 (baseline) and day 2)?
# BNI 0 (baseline) is the reference level to which both INS 2 (day 2
# without shrimp) and IWS 2 (day 2 with shrimp) are compared
# Analysis is done as a one-way anova because the baseline group is the
# same for both INS 2 and IWS 2

> first.lm = lm(Channel_a ~Treat.uni, data=red[red$Day < 3,])
> anova(first.lm)
Analysis of Variance Table

Response: Channel_a
          Df    Sum Sq   Mean Sq F value  Pr(>F)
Treat.uni  2 0.0072222 0.0036111  3.4151 0.04953 *
Residuals 24 0.0253778 0.0010574

> summary(first.lm)
Call:
lm(formula = Channel_a ~ Treat.uni, data = red[red$Day < 3, ])

Coefficients:
             Estimate Std. Error t value Pr(>|t|)
(Intercept)     1.87444    0.01084 172.931   <2e-16 ***
Treat.uniINS 2  0.03889    0.01533   2.537   0.0181 *
Treat.uniIWS 2  0.02778    0.01533   1.812   0.0825 .

Residual standard error: 0.03252 on 24 degrees of freedom
Multiple R-squared:  0.2215,   Adjusted R-squared:  0.1567
F-statistic: 3.415 on 2 and 24 DF,  p-value: 0.04953

## post-hoc tests
> library(multcomp)
> compareFirst = glht(first.lm, linfct=mcp(Treat.uni="Tukey"))
> summary(compareFirst)
```

```
         Simultaneous Tests for General Linear Hypotheses

Multiple Comparisons of Means: Tukey Contrasts

Fit: lm(formula = Channel_a ~ Treat.uni, data = red[red$Day < 3, ])

Linear Hypotheses:
                   Estimate Std. Error t value Pr(>|t|)
INS 2 - BNI 0 == 0  0.03889    0.01533   2.537   0.0458 *
IWS 2 - BNI 0 == 0  0.02778    0.01533   1.812   0.1871
IWS 2 - INS 2 == 0 -0.01111    0.01533  -0.725   0.7513


## B:   Does Shrimp presence affect redness after Day 2?

> later.lm=lm(Channel_a~Shrimp*Day, data=Red[Red$Day>2,])
> anova(later.lm)
Analysis of Variance Table
Response: Channel_a
           Df   Sum Sq   Mean Sq F value   Pr(>F)
Shrimp      1 0.016900 0.0169000  8.3058 0.007004 **
Day         1 0.000044 0.0000444  0.0218 0.883433
Shrimp:Day  1 0.000100 0.0001000  0.0491 0.825963
Residuals     32 0.065111 0.0020347

## rerun omitting interaction term

> later.lm=lm(Channel_a~Shrimp+Day, data=Red[Red$Day>2,])
> summary(later.lm)
Call:
lm(formula = Channel_a ~ Shrimp + Day, data = red[red$Day >
    2, ])

Residuals:
      Min        1Q    Median        3Q       Max
-0.089444 -0.027500 -0.003889  0.028889  0.113889

Coefficients:
                            Estimate Std. Error t value Pr(>|t|)
(Intercept)                 1.895000   0.038498  49.224   <2e-16 ***
ShrimpPresent              -0.043333   0.014818  -2.924   0.0062 **
Day                         0.001111   0.007409   0.150   0.8817

Residual standard error: 0.04445 on 33 degrees of freedom
Multiple R-squared:  0.2062,   Adjusted R-squared:  0.1581
F-statistic: 4.287 on 2 and 33 DF,  p-value: 0.02212


## Analysis of yellowness at injury site (the b-channel for white light)

# C:  Is there a change in yellowness at the injury site
# immediately post-injury(between day 0 (baseline) and day 2)?

>first.lm = lm(formula = channel_b ~ Treat.uni, data=YELLOW[YELLOW$Day <3, ])
> anova(first.lm)
Analysis of Variance Table
Response: channel_b
          Df   Sum Sq   Mean Sq F value Pr(>F)
Treat.uni  2 0.007652 0.0038259  2.4277 0.1096
Residuals 24 0.037822 0.0015759
```

```
# D:   Does Shrimp presence affect yellowness after Day 2?

>later.lm=lm(channel_b~Shrimp+Day,data=YELLOW[YELLOW$Day>2,])
> anova(later.lm)
Analysis of Variance Table
Response: channel_b
           Df   Sum Sq   Mean Sq F value Pr(>F)
Shrimp      1 0.002025 0.0020250  0.6481 0.4266
Day         1 0.001736 0.0017361  0.5556 0.4613
Residuals  33 0.103114 0.0031247
```

Long-wave ultraviolet (tissue damage) analyses

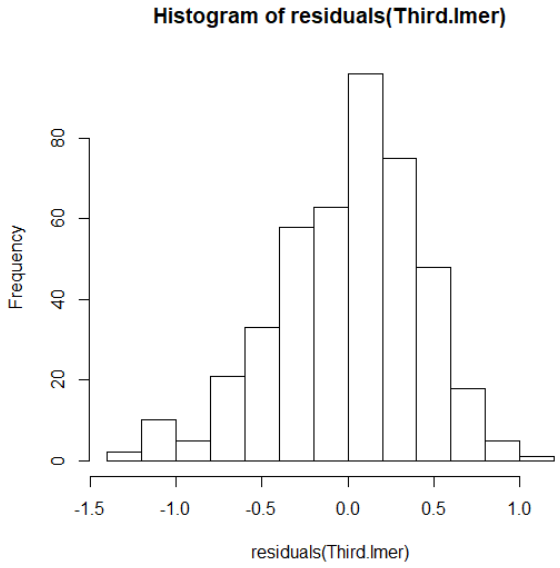
Graphic representation of long-wave UV model:

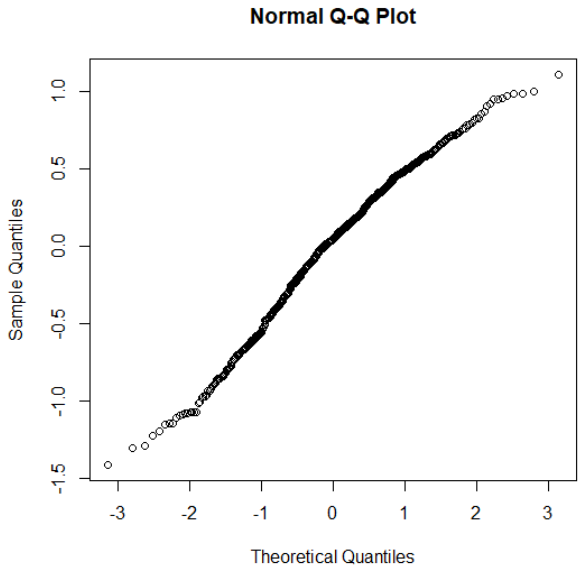


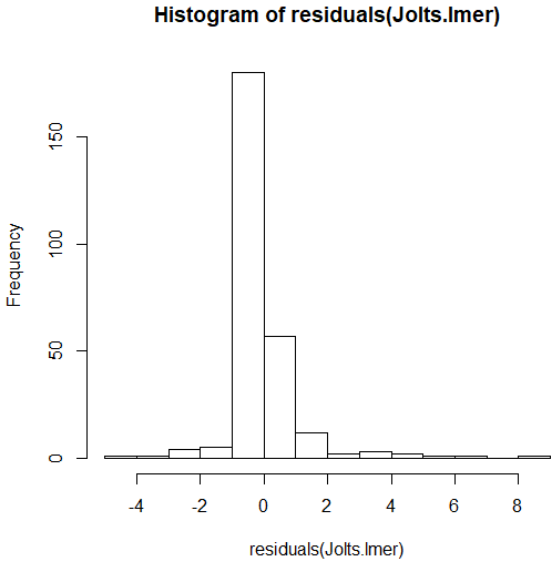

```
# Do shrimp increase levels of non-specific skin injury?

# Analysis of non-specific epithelial injury measured with long-wave
# ultraviolet photography.   Uses packages 'lme4' and 'car'
# Four fixed effects, all analyzed as factors:
# Injured:  fish deliberately injured or not on day 0;
# withShrimp: Shrimp present or absent;
# fday: Day (2, 4, or 6)
# simpSide: Fish side (Injured or control) – uninjured fish had 2
# control sides; injured fish had 1
# Estimable interaction terms all had p-values > 0.10 so only additive
# effects are analyzed below
# Random intercept model with individual fish as a random effect

> UV.lmer = lmer(Area_UV ~ simpSide+fday+withShrimp +Injured +  (1|Fish_UV),
data=UV1)
```

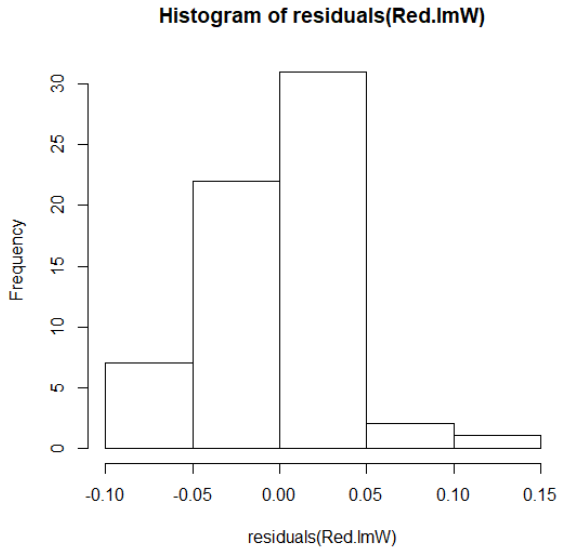
```
> Anova(UV.lmer)
Analysis of Deviance Table (Type II Wald chisquare tests)

Response: Area_UV
            Chisq Df Pr(>Chisq)
simpSide    0.9768  1   0.322983
fday       12.7797  2   0.001679 **
withShrimp  4.1426  1   0.041817 *
Injured     3.5098  1   0.061007 .
```

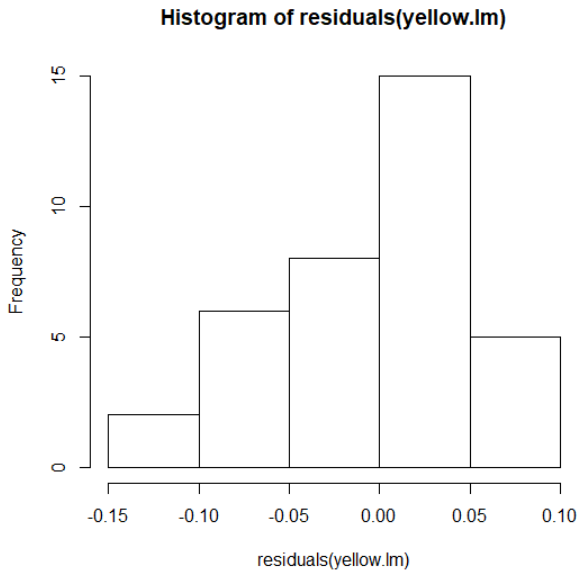
Select diagnostic plots

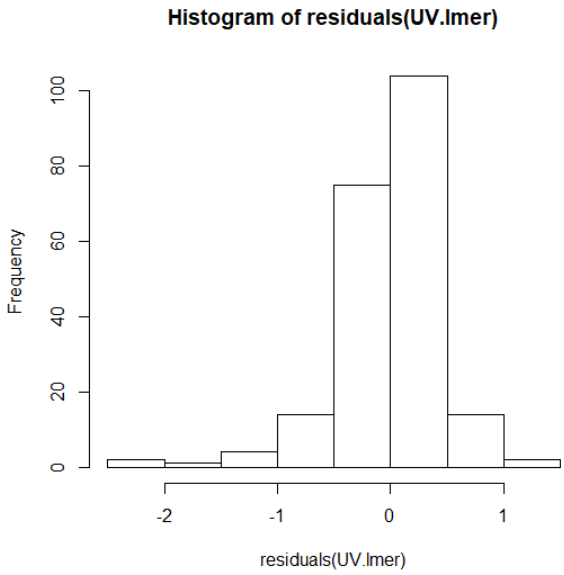






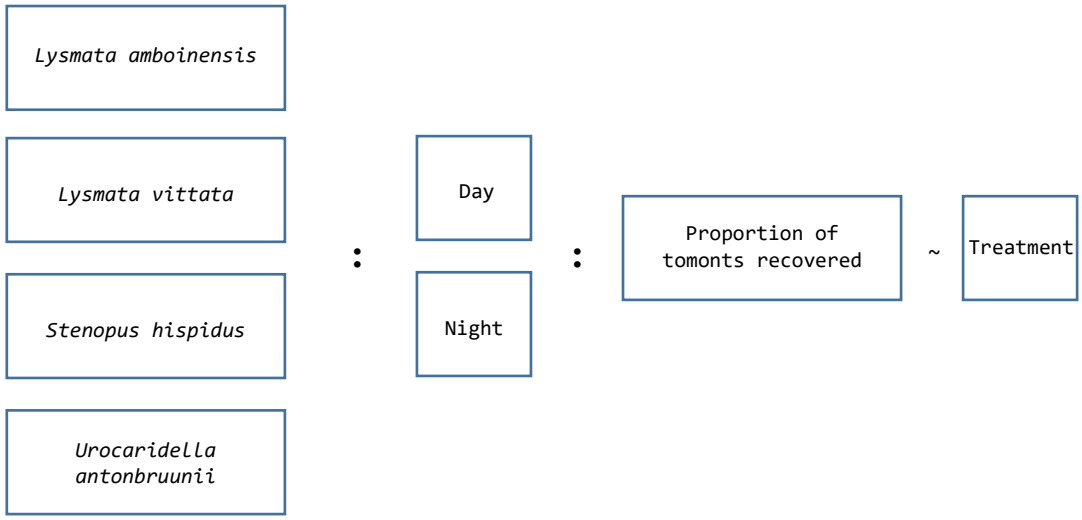



Histogram of residuals(yellow.lm)
Frequency
0
5
10
15
-0.15
-0.10
-0.05
0.00
0.05
0.10
residuals(yellow.lm)


Histogram of residuals(UV.lmer)
Frequency
0
20
40
60
80
100
-2
-1
0
1
residuals(UV.lmer)

Appendix 3. Details of the statistical analyses for Chapter 4 using *R*

Graphic representation of all individual shrimp:ectoparasite models*:



*Within the restriction of the availability of *Zeylanicobdella arugamensis* (see Fig. 4.5), and not performed for *C. irritans* trophonts (on fish) for night data as a function of the lack of treatment significance in the parent model.

CRYPTOCARYON TOMONTS analyses; package='glm2'

#Count data could not be used because the number of tomonts introduced per fish is different between treatment and control (and between all fish), therefore, only the proportional data could be used. So, data are modeled using Quasibinomial regression with a logit link as it was overdispered when analysed with binomial regression.

#Original binomial and quasibinomial models for the dataset are at the end.

Shrimp; LA (*Lysmata amboinensis*), LV (*Lysmata vittata*), SH (*Stenopus hispidus*), UA (*Urocaridella antonbruunii*).

Individual shrimp:*Cryptocaryon irritans* tomonts:

```
LA.day=glm(Tomont_prec_day2~Treatment,family=quasibinomial(link="logit"),data=Tomo
nts.LA)
> summary(LA.day)

Call:
glm(formula = Tomont_prec_day2 ~ Treatment, family = quasibinomial(link =
"logit"),
    data = Tomonts.LA)

Deviance Residuals:
    Min       1Q   Median       3Q      Max
-1.1761  -0.2077   0.1665   0.2810   1.1023
```

```
Coefficients:
                   Estimate Std. Error t value Pr(>|t|)
(Intercept)           4.272      1.708   2.502   0.0222 *
TreatmentTreatment   -4.275      1.753  -2.438   0.0253 *
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for quasibinomial family taken to be 0.395689)

    Null deviance: 16.4206  on 19  degrees of freedom
Residual deviance:  8.9293  on 18  degrees of freedom
AIC: NA

Number of Fisher Scoring iterations: 7

> Anova(LA.day)
Analysis of Deviance Table (Type II tests)

Response: Tomont_prec_day2
          LR Chisq Df Pr(>Chisq)
Treatment   18.932  1  1.354e-05 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
>
>
LA.night=glm(Tomont_prec_night3~Treatment,family=quasibinomial(link="logit"),data=
Tomonts.LA)
> summary(LA.night)

Call:
glm(formula = Tomont_prec_night3 ~ Treatment, family = quasibinomial(link =
"logit"),
    data = Tomonts.LA)

Deviance Residuals:
     Min        1Q    Median        3Q       Max
-0.75292  -0.75292   0.00005   0.00005   1.67279

Coefficients:
                   Estimate Std. Error t value Pr(>|t|)
(Intercept)           20.57    3661.07   0.006    0.996
TreatmentTreatment   -21.68    3661.07  -0.006    0.995

(Dispersion parameter for quasibinomial family taken to be 0.4263643)

    Null deviance: 23.8553  on 19  degrees of freedom
Residual deviance:  8.5367  on 18  degrees of freedom
AIC: NA

Number of Fisher Scoring iterations: 19

> Anova(LA.night)
Analysis of Deviance Table (Type II tests)

Response: Tomont_prec_night3
          LR Chisq Df Pr(>Chisq)
Treatment   35.929  1  2.047e-09 ***
---
```

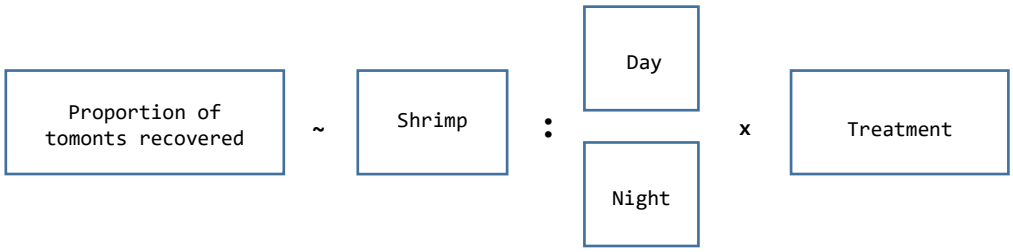
```
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
>
>
LV.day=glm(Tomont_prec_day2~Treatment,family=quasibinomial(link="logit"),data=Tomo
nts.LV)
> summary(LV.day)

Call:
glm(formula = Tomont_prec_day2 ~ Treatment, family = quasibinomial(link =
"logit"),
    data = Tomonts.LV)

Deviance Residuals:
    Min       1Q   Median       3Q      Max
-0.8446  -0.8446   0.1025   0.1025   1.5518

Coefficients:
                  Estimate Std. Error t value Pr(>|t|)
(Intercept)          5.247      3.298   1.591   0.1290
TreatmentTreatment   -6.094      3.338  -1.825   0.0846 .
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for quasibinomial family taken to be 0.5673931)

    Null deviance: 25.478  on 19  degrees of freedom
Residual deviance: 12.387  on 18  degrees of freedom
AIC: NA

Number of Fisher Scoring iterations: 7

> Anova(LV.day)
Analysis of Deviance Table (Type II tests)

Response: Tomont_prec_day2
          LR Chisq Df Pr(>Chisq)
Treatment   23.071  1  1.562e-06 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
>
>
LV.night=glm(Tomont_prec_night3~Treatment,family=quasibinomial(link="logit"),data=
Tomonts.LV)
> summary(LV.night)

Call:
glm(formula = Tomont_prec_night3 ~ Treatment, family = quasibinomial(link =
"logit"),
    data = Tomonts.LV)

Deviance Residuals:
     Min        1Q    Median        3Q       Max
-0.33870  -0.00002  -0.00002   0.20153   0.20153

Coefficients:
                  Estimate Std. Error t value Pr(>|t|)
(Intercept)          3.887      0.395   9.839 1.15e-08 ***
TreatmentTreatment  -26.453   2672.037  -0.010    0.992
---
```

```
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for quasibinomial family taken to be 0.03073692)

    Null deviance: 26.26889  on 19  degrees of freedom
Residual deviance:  0.51976  on 18  degrees of freedom
AIC: NA

Number of Fisher Scoring iterations: 21

> Anova(LV.night)
Analysis of Deviance Table (Type II tests)

Response: Tomont_prec_night3
          LR Chisq Df Pr(>Chisq)
Treatment   837.73  1  < 2.2e-16 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
>
>
SH.day=glm(Tomont_prec_day2~Treatment,family=quasibinomial(link="logit"),data=Tomo
nts.SH)
> summary(SH.day)

Call:
glm(formula = Tomont_prec_day2 ~ Treatment, family = quasibinomial(link =
"logit"),
    data = Tomonts.SH)

Deviance Residuals:
     Min        1Q    Median        3Q       Max
-1.32254  -0.08084   0.09834   0.19454   0.60850

Coefficients:
                  Estimate Std. Error t value Pr(>|t|)
(Intercept)          4.089      1.097   3.729  0.00154 **
TreatmentTreatment  -2.496      1.158  -2.156  0.04490 *
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for quasibinomial family taken to be 0.1948941)

    Null deviance: 4.7519  on 19  degrees of freedom
Residual deviance: 3.1644  on 18  degrees of freedom
AIC: NA

Number of Fisher Scoring iterations: 7

> Anova(SH.day)
Analysis of Deviance Table (Type II tests)

Response: Tomont_prec_day2
          LR Chisq Df Pr(>Chisq)
Treatment   8.1452  1   0.004318 **
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
>
```

```
>
SH.night=glm(Tomont_prec_night3~Treatment,family=quasibinomial(link="logit"),data=
Tomonts.SH)
> summary(SH.night)

Call:
glm(formula = Tomont_prec_night3 ~ Treatment, family = quasibinomial(link =
"logit"),
    data = Tomonts.SH)

Deviance Residuals:
     Min        1Q    Median        3Q       Max
-0.21695  -0.10346   0.08119   0.15944   0.18229

Coefficients:
                   Estimate Std. Error t value Pr(>|t|)
(Intercept)          4.4575     0.4151  10.737 2.96e-09 ***
TreatmentTreatment  -0.3684     0.5411  -0.681    0.505
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for quasibinomial family taken to be 0.01952102)

    Null deviance: 0.43694  on 19  degrees of freedom
Residual deviance: 0.42774  on 18  degrees of freedom
AIC: NA

Number of Fisher Scoring iterations: 7

> Anova(SH.night)
Analysis of Deviance Table (Type II tests)

Response: Tomont_prec_night3
          LR Chisq Df Pr(>Chisq)
Treatment  0.47109  1     0.4925
>
>
UA.day=glm(Tomont_prec_day2~Treatment,family=quasibinomial(link="logit"),data=Tomo
nts.UA)
> summary(UA.day)

Call:
glm(formula = Tomont_prec_day2 ~ Treatment, family = quasibinomial(link =
"logit"),
    data = Tomonts.UA)

Deviance Residuals:
     Min        1Q    Median        3Q       Max
-1.71829  -0.00106   0.19993   0.42577   0.72029

Coefficients:
                   Estimate Std. Error t value Pr(>|t|)
(Intercept)           3.903      1.522   2.563   0.0195 *
TreatmentTreatment   -2.686      1.604  -1.674   0.1113
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for quasibinomial family taken to be 0.4496759)
```

```
    Null deviance: 10.4284  on 19  degrees of freedom
Residual deviance:  8.1179  on 18  degrees of freedom
AIC: NA

Number of Fisher Scoring iterations: 6

> Anova(UA.day)
Analysis of Deviance Table (Type II tests)

Response: Tomont_prec_day2
          LR Chisq Df Pr(>Chisq)
Treatment   5.1381  1    0.02341 *
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
>
>
UA.night=glm(Tomont_prec_night3~Treatment,family=quasibinomial(link="logit"),data=
Tomonts.UA)
> summary(UA.night)

Call:
glm(formula = Tomont_prec_night3 ~ Treatment, family = quasibinomial(link =
"logit"),
    data = Tomonts.UA)

Deviance Residuals:
    Min       1Q   Median       3Q      Max
-1.2166  -0.2894   0.1327   0.3212   1.1387

Coefficients:
                   Estimate Std. Error t value Pr(>|t|)
(Intercept)           4.729      2.369   1.996   0.0612 .
TreatmentTreatment   -4.637      2.410  -1.924   0.0703 .
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for quasibinomial family taken to be 0.4872393)

    Null deviance: 18.943  on 19  degrees of freedom
Residual deviance: 11.613  on 18  degrees of freedom
AIC: NA

Number of Fisher Scoring iterations: 7

> Anova(UA.night)
Analysis of Deviance Table (Type II tests)

Response: Tomont_prec_night3
          LR Chisq Df Pr(>Chisq)
Treatment   15.045  1   0.000105 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
>
```

Graphic representation of all ectoparasite parent models:



#The original model for **DAY** using binomial regression is the following (shows clear overdispersion):

```
>
Original.binomial=glm(cbind(Tomont_rec_day2,Tomont_unrec_day2)~Shrimp*Treatment,fa
mily=binomial(link="logit"),data=Tomonts_correct)
> summary(Original.binomial)

Call:
glm(formula = cbind(Tomont_rec_day2, Tomont_unrec_day2) ~ Shrimp *
    Treatment, family = binomial(link = "logit"), data = Tomonts_correct)

Deviance Residuals:
     Min        1Q    Median        3Q       Max
-11.6054   -1.1674    0.8654    1.7154   12.7635

Coefficients:
                                                 Estimate Std. Error z value
(Intercept)                                        4.2132     0.3808  11.065
ShrimpLysmata_vittata                              1.5489     0.8041   1.926
ShrimpStenopus_hispidus                           -0.2242     0.5218  -0.430
ShrimpUrocaridella_antonbruunii                   -0.3158     0.4876  -0.648
TreatmentTreatment                                -4.2061     0.3900 -10.785
ShrimpLysmata_vittata:TreatmentTreatment          -2.8507     0.8150  -3.498
ShrimpStenopus_hispidus:TreatmentTreatment         1.8530     0.5422   3.418
ShrimpUrocaridella_antonbruunii:TreatmentTreatment 1.4295     0.5060   2.825
                                                  Pr(>|z|)
(Intercept)                                        < 2e-16 ***
ShrimpLysmata_vittata                             0.054072 .
ShrimpStenopus_hispidus                           0.667443
ShrimpUrocaridella_antonbruunii                   0.517159
TreatmentTreatment                                 < 2e-16 ***
ShrimpLysmata_vittata:TreatmentTreatment          0.000469 ***
ShrimpStenopus_hispidus:TreatmentTreatment        0.000632 ***
ShrimpUrocaridella_antonbruunii:TreatmentTreatment 0.004723 **
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for binomial family taken to be 1)

    Null deviance: 3466.7  on 79  degrees of freedom
Residual deviance: 1609.3  on 72  degrees of freedom
AIC: 1728.4

Number of Fisher Scoring iterations: 6
```

```
> Anova(Original.binomial)
Analysis of Deviance Table (Type II tests)

Response: cbind(Tomont_rec_day2, Tomont_unrec_day2)
                LR Chisq Df Pr(>Chisq)
Shrimp            478.02  3  < 2.2e-16 ***
Treatment        1470.83  1  < 2.2e-16 ***
Shrimp:Treatment   70.82  3   2.85e-15 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

#The original model for NIGHT using binomial regression is as follows:

>
Original.binomial2=glm(cbind(Tomont_rec_night2,Tomont_unrec_night2)~Shrimp*Treatme
nt,family=binomial(link="logit"),data=Tomonts_correct)
> summary(Original.binomial2)

Call:
glm(formula = cbind(Tomont_rec_night2, Tomont_unrec_night2) ~
    Shrimp * Treatment, family = binomial(link = "logit"), data = Tomonts_correct)

Deviance Residuals:
     Min        1Q    Median        3Q       Max
-14.6016   -0.9204    0.0005    1.2246   14.2857

Coefficients:
                                                  Estimate Std. Error z value
(Intercept)                                         19.535    479.199   0.041
ShrimpLysmata_vittata                              -15.806    479.199  -0.033
ShrimpStenopus_hispidus                            -15.204    479.199  -0.032
ShrimpUrocaridella_antonbruunii                    -15.079    479.199  -0.031
TreatmentTreatment                                 -20.337    479.199  -0.042
ShrimpLysmata_vittata:TreatmentTreatment            -3.171    677.018  -0.005
ShrimpStenopus_hispidus:TreatmentTreatment          19.995    479.199   0.042
ShrimpUrocaridella_antonbruunii:TreatmentTreatment  16.191    479.199   0.034
                                                  Pr(>|z|)
(Intercept)                                          0.967
ShrimpLysmata_vittata                                0.974
ShrimpStenopus_hispidus                              0.975
ShrimpUrocaridella_antonbruunii                      0.975
TreatmentTreatment                                   0.966
ShrimpLysmata_vittata:TreatmentTreatment             0.996
ShrimpStenopus_hispidus:TreatmentTreatment           0.967
ShrimpUrocaridella_antonbruunii:TreatmentTreatment   0.973

(Dispersion parameter for binomial family taken to be 1)

    Null deviance: 4928.7  on 79  degrees of freedom
Residual deviance: 1331.7  on 72  degrees of freedom
AIC: 1412.6

Number of Fisher Scoring iterations: 15

> Anova(Original.binomial2)
Analysis of Deviance Table (Type II tests)

Response: cbind(Tomont_rec_night2, Tomont_unrec_night2)
```

```
                LR Chisq Df Pr(>Chisq)
Shrimp           1322.34  3  < 2.2e-16 ***
Treatment        2589.97  1  < 2.2e-16 ***
Shrimp:Treatment  148.65  3  < 2.2e-16 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

#Next the quasibinomial **DAY** model (indicates a treatment effect to be investigated further):

```
>
Original.Qbinomial=glm(cbind(Tomont_rec_day2,Tomont_unrec_day2)~Shrimp*Treatment,f
amily=quasibinomial(link="logit"),data=Tomonts_correct)
> summary(Original.Qbinomial)

Call:
glm(formula = cbind(Tomont_rec_day2, Tomont_unrec_day2) ~ Shrimp *
    Treatment, family = quasibinomial(link = "logit"), data = Tomonts_correct)

Deviance Residuals:
     Min        1Q    Median        3Q       Max
-11.6054   -1.1674    0.8654    1.7154   12.7635

Coefficients:
                                                 Estimate Std. Error t value
(Intercept)                                        4.2132     1.7401   2.421
ShrimpLysmata_vittata                              1.5489     3.6748   0.421
ShrimpStenopus_hispidus                           -0.2242     2.3848  -0.094
ShrimpUrocaridella_antonbruunii                   -0.3158     2.2283  -0.142
TreatmentTreatment                                -4.2061     1.7823  -2.360
ShrimpLysmata_vittata:TreatmentTreatment          -2.8507     3.7249  -0.765
ShrimpStenopus_hispidus:TreatmentTreatment         1.8530     2.4779   0.748
ShrimpUrocaridella_antonbruunii:TreatmentTreatment 1.4295     2.3123   0.618
                                                 Pr(>|t|)
(Intercept)                                         0.018 *
ShrimpLysmata_vittata                               0.675
ShrimpStenopus_hispidus                             0.925
ShrimpUrocaridella_antonbruunii                     0.888
TreatmentTreatment                                  0.021 *
ShrimpLysmata_vittata:TreatmentTreatment            0.447
ShrimpStenopus_hispidus:TreatmentTreatment          0.457
ShrimpUrocaridella_antonbruunii:TreatmentTreatment  0.538
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for quasibinomial family taken to be 20.88664)

    Null deviance: 3466.7  on 79  degrees of freedom
Residual deviance: 1609.3  on 72  degrees of freedom
AIC: NA

Number of Fisher Scoring iterations: 6

> Anova(Original.Qbinomial)
Analysis of Deviance Table (Type II tests)

Response: cbind(Tomont_rec_day2, Tomont_unrec_day2)
                LR Chisq Df Pr(>Chisq)
Shrimp            22.886  3  4.265e-05 ***
```

```
Treatment          70.420  1  < 2.2e-16 ***
Shrimp:Treatment    3.391  3     0.3352
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

#Next the Quasibinomial **NIGHT** model (indicates a treatment effect to be investigated further):

```
>
Original.Qbinomial2=glm(cbind(Tomont_rec_night2,Tomont_unrec_night2)~Shrimp*Treatm
ent,family=quasibinomial(link="logit"),data=Tomonts_correct)
> summary(Original.Qbinomial2)

Call:
glm(formula = cbind(Tomont_rec_night2, Tomont_unrec_night2) ~
    Shrimp * Treatment, family = quasibinomial(link = "logit"),
    data = Tomonts_correct)

Deviance Residuals:
     Min        1Q    Median        3Q       Max
-14.6016   -0.9204    0.0005    1.2246   14.2857

Coefficients:
                                                    Estimate Std. Error t value
(Intercept)                                           19.535   1833.513   0.011
ShrimpLysmata_vittata                                -15.806   1833.513  -0.009
ShrimpStenopus_hispidus                              -15.204   1833.513  -0.008
ShrimpUrocaridella_antonbruunii                      -15.079   1833.513  -0.008
TreatmentTreatment                                   -20.337   1833.513  -0.011
ShrimpLysmata_vittata:TreatmentTreatment              -3.171   2590.410  -0.001
ShrimpStenopus_hispidus:TreatmentTreatment            19.995   1833.514   0.011
ShrimpUrocaridella_antonbruunii:TreatmentTreatment    16.191   1833.513   0.009
                                                    Pr(>|t|)
(Intercept)                                            0.992
ShrimpLysmata_vittata                                  0.993
ShrimpStenopus_hispidus                                0.993
ShrimpUrocaridella_antonbruunii                        0.993
TreatmentTreatment                                     0.991
ShrimpLysmata_vittata:TreatmentTreatment               0.999
ShrimpStenopus_hispidus:TreatmentTreatment             0.991
ShrimpUrocaridella_antonbruunii:TreatmentTreatment     0.993

(Dispersion parameter for quasibinomial family taken to be 14.63986)

    Null deviance: 4928.7  on 79  degrees of freedom
Residual deviance: 1331.7  on 72  degrees of freedom
AIC: NA

Number of Fisher Scoring iterations: 15

> Anova(Original.Qbinomial2)
Analysis of Deviance Table (Type II tests)

Response: cbind(Tomont_rec_night2, Tomont_unrec_night2)
                LR Chisq Df Pr(>Chisq)
Shrimp            90.325  3     <2e-16 ***
Treatment        176.912  1     <2e-16 ***
Shrimp:Treatment  10.154  3     0.0173 *
---
```

```
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

CRYPTOCARYON TROPHONTS (on fish; parasitic stage)

```
# DAY:
>
Cryptocaryon.day=glm(cbind(Crypt_rec_day,Crypt_unrec_day)~Shrimp*Treatment,family=
quasibinomial(link="logit"),data=Cryptocaryon)
> summary(Cryptocaryon.day)

Call:
glm(formula = cbind(Crypt_rec_day, Crypt_unrec_day) ~ Shrimp *
    Treatment, family = quasibinomial(link = "logit"), data = Cryptocaryon)

Deviance Residuals:
    Min       1Q   Median       3Q      Max
-8.8478  -2.4794  -0.5394   1.8461   8.4674

Coefficients:
                                                 Estimate Std. Error t value
(Intercept)                                     -1.925946   0.105327 -18.285
ShrimpLysmata_vittata                           -0.079809   0.151248  -0.528
ShrimpStenopus_hispidus                         -0.135509   0.152942  -0.886
ShrimpUrocaridella_antonbruunii                 -0.560695   0.168715  -3.323
TreatmentTreatment                               0.068491   0.147109   0.466
ShrimpLysmata_vittata:TreatmentTreatment        -0.003172   0.211276  -0.015
ShrimpStenopus_hispidus:TreatmentTreatment      -0.365441   0.222580  -1.642
ShrimpUrocaridella_antonbruunii:TreatmentTreatment -0.076984   0.237715  -0.324
                                                Pr(>|t|)
(Intercept)                                       <2e-16 ***
ShrimpLysmata_vittata                             0.5994
ShrimpStenopus_hispidus                           0.3786
ShrimpUrocaridella_antonbruunii                   0.0014 **
TreatmentTreatment                                0.6429
ShrimpLysmata_vittata:TreatmentTreatment          0.9881
ShrimpStenopus_hispidus:TreatmentTreatment        0.1050
ShrimpUrocaridella_antonbruunii:TreatmentTreatment   0.7470
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for quasibinomial family taken to be 12.3164)

    Null deviance: 1322.60  on 79  degrees of freedom
Residual deviance:  892.84  on 72  degrees of freedom
AIC: NA

Number of Fisher Scoring iterations: 4

> library(car)
Warning message:
package ‘car’ was built under R version 3.4.2
> Anova(Cryptocaryon.day)
Analysis of Deviance Table (Type II tests)

Response: cbind(Crypt_rec_day, Crypt_unrec_day)
```

```
                LR Chisq Df Pr(>Chisq)
Shrimp           31.3030  3  7.339e-07 ***
Treatment         0.1610  1     0.6882
Shrimp:Treatment  3.4295  3     0.3300
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
>
>
># NIGHT:
>
Cryptocaryon.night=glm(cbind(Crypt_rec_night,Crypt_unrec_night)~Shrimp*Treatment,f
amily=quasibinomial(link="logit"),data=Cryptocaryon)
> summary(Cryptocaryon.night)

Call:
glm(formula = cbind(Crypt_rec_night, Crypt_unrec_night) ~ Shrimp *
    Treatment, family = quasibinomial(link = "logit"), data = Cryptocaryon)

Deviance Residuals:
     Min        1Q    Median        3Q       Max
-21.4539   -5.4349   -0.8708    3.7332   28.6320

Coefficients:
                                                  Estimate Std. Error t value
(Intercept)                                        0.02120    0.19666   0.108
ShrimpLysmata_vittata                              0.33368    0.28031   1.190
ShrimpStenopus_hispidus                            0.40138    0.28124   1.427
ShrimpUrocaridella_antonbruunii                    0.44676    0.28196   1.584
TreatmentTreatment                                -1.30032    0.30893  -4.209
ShrimpLysmata_vittata:TreatmentTreatment          -0.04614    0.42933  -0.107
ShrimpStenopus_hispidus:TreatmentTreatment         0.83093    0.41780   1.989
ShrimpUrocaridella_antonbruunii:TreatmentTreatment 0.34451    0.42105   0.818
                                                  Pr(>|t|)
(Intercept)                                         0.9145
ShrimpLysmata_vittata                               0.2378
ShrimpStenopus_hispidus                             0.1579
ShrimpUrocaridella_antonbruunii                     0.1175
TreatmentTreatment                                 7.3e-05 ***
ShrimpLysmata_vittata:TreatmentTreatment            0.9147
ShrimpStenopus_hispidus:TreatmentTreatment          0.0505 .
ShrimpUrocaridella_antonbruunii:TreatmentTreatment  0.4159
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for quasibinomial family taken to be 96.67619)

    Null deviance: 14219  on 79  degrees of freedom
Residual deviance:  7334  on 72  degrees of freedom
AIC: NA

Number of Fisher Scoring iterations: 4

> Anova(Cryptocaryon.night)
Analysis of Deviance Table (Type II tests)

Response: cbind(Crypt_rec_night, Crypt_unrec_night)
                LR Chisq Df Pr(>Chisq)
Shrimp            17.359  3  0.0005962 ***
Treatment         48.993  1  2.569e-12 ***
```

```
Shrimp:Treatment    5.874  3  0.1179013
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
>
>
> # No need to test pairwise for day treatments as none were significant in original
day model
>
> # Individual shrimp:Cryptocaryon irritans trophonts:
>
#CF1 = LA; CF2 = LV; CF3 = SH; CF4 = UA
>
Cryptonfish1.night=glm(cbind(Crypt_rec_day,Crypt_unrec_day)~Treatment,family=quasi
binomial(link="logit"),data=Cryptonfish1)
>
Cryptonfish1.night=glm(cbind(Crypt_rec_night,Crypt_unrec_night)~Treatment,family=q
uasibinomial(link="logit"),data=Cryptonfish1)
> summary(Cryptonfish1.night)

Call:
glm(formula = cbind(Crypt_rec_night, Crypt_unrec_night) ~ Treatment,
    family = quasibinomial(link = "logit"), data = Cryptonfish1)

Deviance Residuals:
    Min       1Q   Median       3Q      Max
-21.454  -10.769   -2.078    9.238   26.416

Coefficients:
                  Estimate Std. Error t value Pr(>|t|)
(Intercept)         0.0212     0.2516   0.084  0.93378
TreatmentTreatment  -1.3003     0.3953  -3.290  0.00407 **
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for quasibinomial family taken to be 158.2571)

    Null deviance: 4900.8  on 19  degrees of freedom
Residual deviance: 3071.2  on 18  degrees of freedom
AIC: NA

Number of Fisher Scoring iterations: 4

> Anova(Cryptonfish1.night)
Analysis of Deviance Table (Type II tests)

Response: cbind(Crypt_rec_night, Crypt_unrec_night)
          LR Chisq Df Pr(>Chisq)
Treatment   11.561  1  0.0006735 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
>
>
Cryptonfish2.night=glm(cbind(Crypt_rec_night,Crypt_unrec_night)~Treatment,family=q
uasibinomial(link="logit"),data=Cryptonfish2)
> summary(Cryptonfish2.night)

Call:
glm(formula = cbind(Crypt_rec_night, Crypt_unrec_night) ~ Treatment,
    family = quasibinomial(link = "logit"), data = Cryptonfish2)
```

```
Deviance Residuals:
    Min       1Q   Median       3Q      Max
-19.233   -8.147   -4.179    6.182   28.632

Coefficients:
                   Estimate Std. Error t value Pr(>|t|)
(Intercept)          0.3549     0.2739   1.295  0.21154
TreatmentTreatment  -1.3465     0.4089  -3.293  0.00404 **
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for quasibinomial family taken to be 181.8273)

    Null deviance: 5496.5  on 19  degrees of freedom
Residual deviance: 3403.1  on 18  degrees of freedom
AIC: NA

Number of Fisher Scoring iterations: 4

> Anova(Cryptonfish2.night)
Analysis of Deviance Table (Type II tests)

Response: cbind(Crypt_rec_night, Crypt_unrec_night)
          LR Chisq Df Pr(>Chisq)
Treatment   11.513  1   0.000691 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
>
>
Cryptonfish3.night=glm(cbind(Crypt_rec_night,Crypt_unrec_night)~Treatment,family=q
uasibinomial(link="logit"),data=Cryptonfish3)
> summary(Cryptonfish3.night)

Call:
glm(formula = cbind(Crypt_rec_night, Crypt_unrec_night) ~ Treatment,
    family = quasibinomial(link = "logit"), data = Cryptonfish3)

Deviance Residuals:
    Min       1Q   Median       3Q      Max
-9.6060  -1.6165   0.5633   3.2235   7.3941

Coefficients:
                   Estimate Std. Error t value Pr(>|t|)
(Intercept)         0.42258    0.09971   4.238 0.000494 ***
TreatmentTreatment -0.46939    0.13949  -3.365 0.003448 **
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for quasibinomial family taken to be 23.77648)

    Null deviance: 704.81  on 19  degrees of freedom
Residual deviance: 433.67  on 18  degrees of freedom
AIC: NA

Number of Fisher Scoring iterations: 3

> Anova(Cryptonfish3.night)
Analysis of Deviance Table (Type II tests)
```

```
Response: cbind(Crypt_rec_night, Crypt_unrec_night)
          LR Chisq Df Pr(>Chisq)
Treatment   11.404  1   0.000733 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
>
>
Cryptonfish4.night=glm(cbind(Crypt_rec_night,Crypt_unrec_night)~Treatment,family=q
uasibinomial(link="logit"),data=Cryptonfish4)
> summary(Cryptonfish4.night)

Call:
glm(formula = cbind(Crypt_rec_night, Crypt_unrec_night) ~ Treatment,
    family = quasibinomial(link = "logit"), data = Cryptonfish4)

Deviance Residuals:
    Min       1Q   Median       3Q      Max
-6.7301  -3.2839  -0.5617   1.9995  11.6676

Coefficients:
                   Estimate Std. Error t value Pr(>|t|)
(Intercept)         0.46796    0.09822   4.764 0.000155 ***
TreatmentTreatment -0.95581    0.13907  -6.873 1.98e-06 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for quasibinomial family taken to be 22.84395)

    Null deviance: 1536.1  on 19  degrees of freedom
Residual deviance:  426.0  on 18  degrees of freedom
AIC: NA

Number of Fisher Scoring iterations: 4

> Anova(Cryptonfish4.night)
Analysis of Deviance Table (Type II tests)

Response: cbind(Crypt_rec_night, Crypt_unrec_night)
          LR Chisq Df Pr(>Chisq)
Treatment   48.597  1  3.144e-12 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

***NEOBENEDENIA GIRELLAE* (on fish; parasitic stage)**

 # DAY:

```
DayQ=glm(cbind(Recovered_day,Unrecovered_day)~Shrimp*Treatment,family=quasibinomia
l(link="logit"),data=Neo)
> summary(DayQ)

Call:
glm(formula = cbind(Recovered_day, Unrecovered_day) ~ Shrimp *
    Treatment, family = quasibinomial(link = "logit"), data = Neo)

Deviance Residuals:
```

```
    Min       1Q   Median       3Q      Max
-5.7170  -1.6085  -0.1097   1.5660   6.6048

Coefficients:
                                                    Estimate Std. Error t value
(Intercept)                                          1.04597    0.33549   3.118
ShrimpLysmata_vittata                               -1.13937    0.44650  -2.552
ShrimpStenopus_hispidus                             -0.77770    0.44804  -1.736
ShrimpUrocaridella_antonbruunii                      0.23923    0.49004   0.488
TreatmentTreatment                                  -1.36874    0.44883  -3.050
ShrimpLysmata_vittata:TreatmentTreatment             1.02882    0.61564   1.671
ShrimpStenopus_hispidus:TreatmentTreatment           1.53380    0.61676   2.487
ShrimpUrocaridella_antonbruunii:TreatmentTreatment   0.01685    0.64480   0.026
                                                    Pr(>|t|)
(Intercept)                                          0.00262 **
ShrimpLysmata_vittata                                0.01284 *
ShrimpStenopus_hispidus                              0.08688 .
ShrimpUrocaridella_antonbruunii                      0.62691
TreatmentTreatment                                   0.00321 **
ShrimpLysmata_vittata:TreatmentTreatment             0.09904 .
ShrimpStenopus_hispidus:TreatmentTreatment           0.01521 *
ShrimpUrocaridella_antonbruunii:TreatmentTreatment   0.97922
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for quasibinomial family taken to be 6.496779)

    Null deviance: 750.34  on 79  degrees of freedom
Residual deviance: 567.63  on 72  degrees of freedom
AIC: NA

Number of Fisher Scoring iterations: 4

> Anova(DayQ)
Analysis of Deviance Table (Type II tests)

Response: cbind(Recovered_day, Unrecovered_day)
                LR Chisq Df Pr(>Chisq)
Shrimp            8.5073  3   0.036613 *
Treatment        10.5959  1   0.001133 **
Shrimp:Treatment  9.2624  3   0.025998 *
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
>
>
# NIGHT:
>
NightQ=glm(cbind(Recovered_night,Unrecovered_night)~Shrimp*Treatment,family=quasib
inomial(link="logit"),data=Neo)
> summary(NightQ)

Call:
glm(formula = cbind(Recovered_night, Unrecovered_night) ~ Shrimp *
    Treatment, family = quasibinomial(link = "logit"), data = Neo)

Deviance Residuals:
    Min       1Q   Median       3Q      Max
-6.1239  -1.6931  -0.3479   1.2679   8.2684
```

```
Coefficients:
                                                  Estimate Std. Error t value
(Intercept)                                       -0.05335    0.28267  -0.189
ShrimpLysmata_vittata                              0.28099    0.40099   0.701
ShrimpStenopus_hispidus                            1.26166    0.43889   2.875
ShrimpUrocaridella_antonbruunii                    0.47272    0.40412   1.170
TreatmentTreatment                                -1.76194    0.49568  -3.555
ShrimpLysmata_vittata:TreatmentTreatment           0.78053    0.64678   1.207
ShrimpStenopus_hispidus:TreatmentTreatment         0.71398    0.66240   1.078
ShrimpUrocaridella_antonbruunii:TreatmentTreatment 0.54245    0.64995   0.835
                                                  Pr(>|t|)
(Intercept)                                       0.850844
ShrimpLysmata_vittata                             0.485720
ShrimpStenopus_hispidus                           0.005314 **
ShrimpUrocaridella_antonbruunii                   0.245962
TreatmentTreatment                                0.000673 ***
ShrimpLysmata_vittata:TreatmentTreatment          0.231463
ShrimpStenopus_hispidus:TreatmentTreatment        0.284697
ShrimpUrocaridella_antonbruunii:TreatmentTreatment 0.406701
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for quasibinomial family taken to be 5.988584)

    Null deviance: 841.09  on 79  degrees of freedom
Residual deviance: 483.98  on 72  degrees of freedom
AIC: NA

Number of Fisher Scoring iterations: 5

> Anova(NightQ)
Analysis of Deviance Table (Type II tests)

Response: cbind(Recovered_night, Unrecovered_night)
               LR Chisq Df Pr(>Chisq)
Shrimp           26.657  3  6.946e-06 ***
Treatment        33.388  1  7.550e-09 ***
Shrimp:Treatment  1.739  3     0.6282
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
>
>
> # Individual shrimp:Neobenedenia girellae on fish:
>
#OF1 = LA; OF2 = LV; OF3 = SH; OF4 = UA.
>
# DAY:
>
>Onfish1.day=glm(cbind(Recovered_day,Unrecovered_day)~Treatment,family=quasibinomi
al(link="logit"),data=Onfish1)
> summary(Onfish1.day)

Call:
glm(formula = cbind(Recovered_day, Unrecovered_day) ~ Treatment,
    family = quasibinomial(link = "logit"), data = Onfish1)

Deviance Residuals:
    Min       1Q   Median       3Q      Max
-5.7170  -1.8596  -0.2241   2.4492   5.5325
```

```
Coefficients:
                   Estimate Std. Error t value Pr(>|t|)
(Intercept)          1.0460     0.3919   2.669   0.0157 *
TreatmentTreatment  -1.3687     0.5244  -2.610   0.0177 *
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for quasibinomial family taken to be 8.866971)

    Null deviance: 262.08  on 19  degrees of freedom
Residual deviance: 197.74  on 18  degrees of freedom
AIC: NA

Number of Fisher Scoring iterations: 4

> Anova(Onfish1.day)
Analysis of Deviance Table (Type II tests)

Response: cbind(Recovered_day, Unrecovered_day)
          LR Chisq Df Pr(>Chisq)
Treatment   7.2563  1   0.007065 **
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
>
# NIGHT:
>
Onfish1.night=glm(cbind(Recovered_night,Unrecovered_night)~Treatment,family=quasib
inomial(link="logit"),data=Onfish1)
> summary(Onfish1.night)

Call:
glm(formula = cbind(Recovered_night, Unrecovered_night) ~ Treatment,
    family = quasibinomial(link = "logit"), data = Onfish1)

Deviance Residuals:
    Min       1Q   Median       3Q      Max
-3.0082  -1.4877  -1.2975   0.8828   6.5735

Coefficients:
                   Estimate Std. Error t value Pr(>|t|)
(Intercept)        -0.05335    0.29530  -0.181  0.85866
TreatmentTreatment -1.76194    0.51782  -3.403  0.00317 **
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for quasibinomial family taken to be 6.535413)

    Null deviance: 215.50  on 19  degrees of freedom
Residual deviance: 128.06  on 18  degrees of freedom
AIC: NA

Number of Fisher Scoring iterations: 5

> Anova(Onfish1.night)
Analysis of Deviance Table (Type II tests)

Response: cbind(Recovered_night, Unrecovered_night)
          LR Chisq Df Pr(>Chisq)
```

```
Treatment   13.379  1  0.0002545 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
>
#Run both day and night for Lysmata vittata with quasibinomial regression and
logit link:
>
# DAY:
>
Onfish2.day=glm(cbind(Recovered_day,Unrecovered_day)~Treatment,family=quasibinomia
l(link="logit"),data=Onfish2)
> summary(Onfish2.day)

Call:
glm(formula = cbind(Recovered_day, Unrecovered_day) ~ Treatment,
    family = quasibinomial(link = "logit"), data = Onfish2)

Deviance Residuals:
    Min       1Q   Median       3Q      Max
-5.4760  -1.1027  -0.1097   0.7125   3.0214

Coefficients:
                  Estimate Std. Error t value Pr(>|t|)
(Intercept)        -0.0934     0.2209  -0.423    0.677
TreatmentTreatment  -0.3399     0.3159  -1.076    0.296

(Dispersion parameter for quasibinomial family taken to be 3.651915)

    Null deviance: 80.687  on 19  degrees of freedom
Residual deviance: 76.443  on 18  degrees of freedom
AIC: NA

Number of Fisher Scoring iterations: 4

> Anova(Onfish2.day)
Analysis of Deviance Table (Type II tests)

Response: cbind(Recovered_day, Unrecovered_day)
          LR Chisq Df Pr(>Chisq)
Treatment    1.162  1      0.281
>
# NIGHT:
>
Onfish2.night=glm(cbind(Recovered_night,Unrecovered_night)~Treatment,family=quasib
inomial(link="logit"),data=Onfish2)
> summary(Onfish2.night)

Call:
glm(formula = cbind(Recovered_night, Unrecovered_night) ~ Treatment,
    family = quasibinomial(link = "logit"), data = Onfish2)

Deviance Residuals:
    Min       1Q   Median       3Q      Max
-3.9848  -1.9089  -0.8124   0.8695   8.2684

Coefficients:
                  Estimate Std. Error t value Pr(>|t|)
(Intercept)         0.2276     0.3337   0.682   0.5038
TreatmentTreatment  -0.9814     0.4875  -2.013   0.0593 .
```

```
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for quasibinomial family taken to be 8.245501)

    Null deviance: 204.35  on 19  degrees of freedom
Residual deviance: 169.87  on 18  degrees of freedom
AIC: NA

Number of Fisher Scoring iterations: 4

> Anova(Onfish2.night)
Analysis of Deviance Table (Type II tests)

Response: cbind(Recovered_night, Unrecovered_night)
          LR Chisq Df Pr(>Chisq)
Treatment   4.1815  1    0.04087 *
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
>
#Run both day and night for Stenopus hispidus with quasibinomial regression and
logit link:
>
# DAY:
>
Onfish3.day=glm(cbind(Recovered_day,Unrecovered_day)~Treatment,family=quasibinomia
l(link="logit"),data=Onfish3)
> summary(Onfish3.day)

Call:
glm(formula = cbind(Recovered_day, Unrecovered_day) ~ Treatment,
    family = quasibinomial(link = "logit"), data = Onfish3)

Deviance Residuals:
    Min       1Q   Median       3Q      Max
-3.3962  -1.2114  -0.2211   1.2041   5.4760

Coefficients:
                   Estimate Std. Error t value Pr(>|t|)
(Intercept)          0.2683     0.2539   1.057    0.305
TreatmentTreatment   0.1651     0.3616   0.456    0.654

(Dispersion parameter for quasibinomial family taken to be 4.747627)

    Null deviance: 102.66  on 19  degrees of freedom
Residual deviance: 101.67  on 18  degrees of freedom
AIC: NA

Number of Fisher Scoring iterations: 4

> Anova(Onfish3.day)
Analysis of Deviance Table (Type II tests)

Response: cbind(Recovered_day, Unrecovered_day)
          LR Chisq Df Pr(>Chisq)
Treatment  0.20853  1     0.6479
```

```
# NIGHT:
>
Onfish3.night=glm(cbind(Recovered_night,Unrecovered_night)~Treatment,family=quasib
inomial(link="logit"),data=Onfish3)
> summary(Onfish3.night)

Call:
glm(formula = cbind(Recovered_night, Unrecovered_night) ~ Treatment,
    family = quasibinomial(link = "logit"), data = Onfish3)

Deviance Residuals:
    Min       1Q   Median       3Q      Max
-6.1239  -1.0465   0.4779   1.8056   3.0545

Coefficients:
                  Estimate Std. Error t value Pr(>|t|)
(Intercept)         1.2083     0.2857   4.229 0.000504 ***
TreatmentTreatment  -1.0480     0.3739  -2.803 0.011770 *
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for quasibinomial family taken to be 4.336604)

    Null deviance: 122.771  on 19  degrees of freedom
Residual deviance:  87.148  on 18  degrees of freedom
AIC: NA

Number of Fisher Scoring iterations: 4

> Anova(Onfish3.night)
Analysis of Deviance Table (Type II tests)

Response: cbind(Recovered_night, Unrecovered_night)
          LR Chisq Df Pr(>Chisq)
Treatment   8.2146  1   0.004155 **
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
>
#Run both day and night for Urocaridella antonbruunii with quasibinomial
regression and logit link:
>
# DAY:
>
>Onfish4.day=glm(cbind(Recovered_day,Unrecovered_day)~Treatment,family=quasibinomi
al(link="logit"),data=Onfish4)
> summary(Onfish4.day)

Call:
glm(formula = cbind(Recovered_day, Unrecovered_day) ~ Treatment,
    family = quasibinomial(link = "logit"), data = Onfish4)

Deviance Residuals:
    Min       1Q   Median       3Q      Max
-5.5665  -1.9130  -0.0006   1.6057   6.6048

Coefficients:
                  Estimate Std. Error t value Pr(>|t|)
(Intercept)         1.2852     0.4138   3.106  0.00611 **
TreatmentTreatment  -1.3519     0.5364  -2.521  0.02137 *
```

```
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for quasibinomial family taken to be 8.720601)

    Null deviance: 251.22  on 19  degrees of freedom
Residual deviance: 191.78  on 18  degrees of freedom
AIC: NA

Number of Fisher Scoring iterations: 4

> Anova(Onfish4.day)
Analysis of Deviance Table (Type II tests)

Response: cbind(Recovered_day, Unrecovered_day)
          LR Chisq Df Pr(>Chisq)
Treatment   6.8161  1   0.009034 **
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
>
# NIGHT:
>
Onfish4.night=glm(cbind(Recovered_night,Unrecovered_night)~Treatment,family=quasib
inomial(link="logit"),data=Onfish4)
> summary(Onfish4.night)

Call:
glm(formula = cbind(Recovered_night, Unrecovered_night) ~ Treatment,
    family = quasibinomial(link = "logit"), data = Onfish4)

Deviance Residuals:
    Min       1Q   Median       3Q      Max
-4.7185  -1.3076   0.1186   1.5686   3.2692

Coefficients:
                  Estimate Std. Error t value Pr(>|t|)
(Intercept)         0.4194     0.2596   1.616  0.12354
TreatmentTreatment  -1.2195     0.3778  -3.228  0.00467 **
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for quasibinomial family taken to be 4.836866)

    Null deviance: 151.72  on 19  degrees of freedom
Residual deviance:  98.90  on 18  degrees of freedom
AIC: NA

Number of Fisher Scoring iterations: 4

> Anova(Onfish4.night)
Analysis of Deviance Table (Type II tests)

Response: cbind(Recovered_night, Unrecovered_night)
          LR Chisq Df Pr(>Chisq)
Treatment   10.921  1   0.000951 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
>
```

***NEOBENEDENIA GIRELLAE* EGGS**

```
#Original binomial models on complete dataset (showing overdispersion):

# DAY:
>
Original.eggs.glm=glm(cbind(Eggs_recovered_day,Eggs_unrecovered_day)~Shrimp*Treatm
ent,family=binomial(link="logit"),data=Neo.eggs2)
> summary(Original.eggs.glm)

Call:
glm(formula = cbind(Eggs_recovered_day, Eggs_unrecovered_day) ~
    Shrimp * Treatment, family = binomial(link = "logit"), data = Neo.eggs2)

Deviance Residuals:
    Min       1Q   Median       3Q      Max
-13.207   -2.390    1.294    1.859   10.269

Coefficients:
                                                  Estimate Std. Error z value
(Intercept)                                         3.5766     0.2927  12.220
ShrimpLysmata_vittata                               0.2054     0.4335   0.474
ShrimpStenopus_hispidus                             0.4247     0.4459   0.953
ShrimpUrocaridella_antonbruunii                     0.4839     0.4457   1.086
TreatmentTreatment                                 -3.4492     0.3056 -11.286
ShrimpLysmata_vittata:TreatmentTreatment           -1.5892     0.4548  -3.494
ShrimpStenopus_hispidus:TreatmentTreatment          0.1582     0.4638   0.341
ShrimpUrocaridella_antonbruunii:TreatmentTreatment  1.1920     0.4718   2.527
                                                  Pr(>|z|)
(Intercept)                                        < 2e-16 ***
ShrimpLysmata_vittata                             0.635697
ShrimpStenopus_hispidus                           0.340836
ShrimpUrocaridella_antonbruunii                   0.277656
TreatmentTreatment                                 < 2e-16 ***
ShrimpLysmata_vittata:TreatmentTreatment          0.000475 ***
ShrimpStenopus_hispidus:TreatmentTreatment        0.733038
ShrimpUrocaridella_antonbruunii:TreatmentTreatment 0.011519 *
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for binomial family taken to be 1)

    Null deviance: 3551.0  on 79  degrees of freedom
Residual deviance: 1945.3  on 72  degrees of freedom
AIC: 2050.9

Number of Fisher Scoring iterations: 5

> Anova(Original.eggs.glm)
Analysis of Deviance Table (Type II tests)

Response: cbind(Eggs_recovered_day, Eggs_unrecovered_day)
               LR Chisq Df Pr(>Chisq)
Shrimp           452.94  3  < 2.2e-16 ***
Treatment       1193.23  1  < 2.2e-16 ***
Shrimp:Treatment   34.05  3  1.934e-07 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

NIGHT:

```
>Original.eggs.glm2=glm(cbind(Eggs_recovered_night,Eggs_unrecovered_night)~Shrimp*
Treatment,family=binomial(link="logit"),data=Neo.eggs2)
> summary(Original.eggs.glm2)

Call:
glm(formula = cbind(Eggs_recovered_night, Eggs_unrecovered_night) ~
    Shrimp * Treatment, family = binomial(link = "logit"), data = Neo.eggs2)

Deviance Residuals:
    Min       1Q   Median       3Q      Max
-15.186   -2.804    0.970    1.572   12.416

Coefficients:
                                                  Estimate Std. Error z value
(Intercept)                                        4.53689    0.44960  10.091
ShrimpLysmata_vittata                             -0.03523    0.60882  -0.058
ShrimpStenopus_hispidus                           -0.22609    0.60913  -0.371
ShrimpUrocaridella_antonbruunii                   -0.16323    0.60902  -0.268
TreatmentTreatment                                -6.09828    0.46592 -13.089
ShrimpLysmata_vittata:TreatmentTreatment          -0.38493    0.63534  -0.606
ShrimpStenopus_hispidus:TreatmentTreatment         2.62215    0.62902   4.169
ShrimpUrocaridella_antonbruunii:TreatmentTreatment 3.92595    0.63751   6.158
                                                  Pr(>|z|)
(Intercept)                                        < 2e-16 ***
ShrimpLysmata_vittata                                0.954
ShrimpStenopus_hispidus                              0.711
ShrimpUrocaridella_antonbruunii                      0.789
TreatmentTreatment                                 < 2e-16 ***
ShrimpLysmata_vittata:TreatmentTreatment             0.545
ShrimpStenopus_hispidus:TreatmentTreatment        3.06e-05 ***
ShrimpUrocaridella_antonbruunii:TreatmentTreatment 7.35e-10 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for binomial family taken to be 1)

    Null deviance: 4250.5  on 79  degrees of freedom
Residual deviance: 1641.8  on 72  degrees of freedom
AIC: 1707.9

Number of Fisher Scoring iterations: 7

> Anova(Original.eggs.glm2)
Analysis of Deviance Table (Type II tests)

Response: cbind(Eggs_recovered_night, Eggs_unrecovered_night)
                LR Chisq Df Pr(>Chisq)
Shrimp            979.28  3  < 2.2e-16 ***
Treatment        1966.09  1  < 2.2e-16 ***
Shrimp:Treatment   49.91  3  8.365e-11 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
>

#Original models using quasibinomial regression and logit link:
```

DAY:

```
>
Original.eggs.Qglm=glm(cbind(Eggs_recovered_day,Eggs_unrecovered_day)~Shrimp*Treat
ment,family=quasibinomial(link="logit"),data=Neo.eggs2)
> summary(Original.eggs.Qglm)

Call:
glm(formula = cbind(Eggs_recovered_day, Eggs_unrecovered_day) ~
    Shrimp * Treatment, family = quasibinomial(link = "logit"),
    data = Neo.eggs2)

Deviance Residuals:
    Min       1Q   Median       3Q      Max
-13.207   -2.390    1.294    1.859   10.269

Coefficients:
                                                 Estimate Std. Error t value
(Intercept)                                        3.5766     1.4433   2.478
ShrimpLysmata_vittata                              0.2054     2.1378   0.096
ShrimpStenopus_hispidus                            0.4247     2.1988   0.193
ShrimpUrocaridella_antonbruunii                    0.4839     2.1981   0.220
TreatmentTreatment                                -3.4492     1.5071  -2.289
ShrimpLysmata_vittata:TreatmentTreatment          -1.5892     2.2428  -0.709
ShrimpStenopus_hispidus:TreatmentTreatment         0.1582     2.2872   0.069
ShrimpUrocaridella_antonbruunii:TreatmentTreatment 1.1920     2.3266   0.512
                                                 Pr(>|t|)
(Intercept)                                        0.0156 *
ShrimpLysmata_vittata                              0.9237
ShrimpStenopus_hispidus                            0.8474
ShrimpUrocaridella_antonbruunii                    0.8264
TreatmentTreatment                                 0.0250 *
ShrimpLysmata_vittata:TreatmentTreatment           0.4809
ShrimpStenopus_hispidus:TreatmentTreatment         0.9450
ShrimpUrocaridella_antonbruunii:TreatmentTreatment 0.6100
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for quasibinomial family taken to be 24.31809)

    Null deviance: 3551.0  on 79  degrees of freedom
Residual deviance: 1945.3  on 72  degrees of freedom
AIC: NA

Number of Fisher Scoring iterations: 5

> Anova(Original.eggs.Qglm)
Analysis of Deviance Table (Type II tests)

Response: cbind(Eggs_recovered_day, Eggs_unrecovered_day)
               LR Chisq Df Pr(>Chisq)
Shrimp           18.626  3  0.0003267 ***
Treatment        49.068  1  2.473e-12 ***
Shrimp:Treatment  1.400  3  0.7054924
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
>
```

NIGHT:

```
>
Original.eggs.Qglm2=glm(cbind(Eggs_recovered_night,Eggs_unrecovered_night)~Shrimp*
Treatment,family=quasibinomial(link="logit"),data=Neo.eggs2)
> summary(Original.eggs.Qglm2)

Call:
glm(formula = cbind(Eggs_recovered_night, Eggs_unrecovered_night) ~
    Shrimp * Treatment, family = quasibinomial(link = "logit"),
    data = Neo.eggs2)

Deviance Residuals:
    Min       1Q   Median       3Q      Max
-15.186   -2.804    0.970    1.572   12.416

Coefficients:
                                                  Estimate Std. Error t value
(Intercept)                                        4.53689    2.28589   1.985
ShrimpLysmata_vittata                             -0.03523    3.09538  -0.011
ShrimpStenopus_hispidus                           -0.22609    3.09700  -0.073
ShrimpUrocaridella_antonbruunii                   -0.16323    3.09643  -0.053
TreatmentTreatment                                -6.09828    2.36884  -2.574
ShrimpLysmata_vittata:TreatmentTreatment          -0.38493    3.23022  -0.119
ShrimpStenopus_hispidus:TreatmentTreatment         2.62215    3.19812   0.820
ShrimpUrocaridella_antonbruunii:TreatmentTreatment 3.92595    3.24124   1.211
                                                  Pr(>|t|)
(Intercept)                                         0.0510 .
ShrimpLysmata_vittata                               0.9910
ShrimpStenopus_hispidus                             0.9420
ShrimpUrocaridella_antonbruunii                     0.9581
TreatmentTreatment                                  0.0121 *
ShrimpLysmata_vittata:TreatmentTreatment            0.9055
ShrimpStenopus_hispidus:TreatmentTreatment          0.4150
ShrimpUrocaridella_antonbruunii:TreatmentTreatment  0.2298
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for quasibinomial family taken to be 25.84974)

    Null deviance: 4250.5  on 79  degrees of freedom
Residual deviance: 1641.8  on 72  degrees of freedom
AIC: NA

Number of Fisher Scoring iterations: 7

> Anova(Original.eggs.Qglm2)
Analysis of Deviance Table (Type II tests)

Response: cbind(Eggs_recovered_night, Eggs_unrecovered_night)
               LR Chisq Df Pr(>Chisq)
Shrimp           37.884  3  2.991e-08 ***
Treatment        76.058  1  < 2.2e-16 ***
Shrimp:Treatment  1.931  3     0.5869
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
>
>
# Pairwise per shrimp species:
>
>
```

```
# DAY:
>
Eggs.LA.day=glm(P_eggs_recovered_day~Treatment,family=quasibinomial(link="logit"),
data=Eggs.LA)
> summary(Eggs.LA.day)

Call:
glm(formula = P_eggs_recovered_day ~ Treatment, family = quasibinomial(link =
"logit"),
    data = Eggs.LA)

Deviance Residuals:
    Min       1Q   Median       3Q      Max
-1.1466  -0.5942   0.2373   0.3420   1.2085

Coefficients:
                  Estimate Std. Error t value Pr(>|t|)
(Intercept)          3.556      1.416   2.512   0.0218 *
TreatmentTreatment  -3.629      1.490  -2.435   0.0255 *
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for quasibinomial family taken to be 0.5408711)

    Null deviance: 19.892  on 19  degrees of freedom
Residual deviance: 12.831  on 18  degrees of freedom
AIC: NA

Number of Fisher Scoring iterations: 6

> Anova(Eggs.LA.day)
Analysis of Deviance Table (Type II tests)

Response: P_eggs_recovered_day
          LR Chisq Df Pr(>Chisq)
Treatment   13.055  1  0.0003024 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
>
# NIGHT:
>
Eggs.LA.night=glm(P_eggs_recovered_night~Treatment,family=quasibinomial(link="logi
t"),data=Eggs.LA)
> summary(Eggs.LA.night)

Call:
glm(formula = P_eggs_recovered_night ~ Treatment, family = quasibinomial(link =
"logit"),
    data = Eggs.LA)

Deviance Residuals:
     Min        1Q    Median        3Q       Max
-0.66805  -0.66805   0.00946   0.13536   1.79412

Coefficients:
                  Estimate Std. Error t value Pr(>|t|)
(Intercept)          4.688      2.539   1.846   0.0814 .
TreatmentTreatment  -6.075      2.610  -2.327   0.0318 *
---
```

```
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for quasibinomial family taken to be 0.5827241)

    Null deviance: 26.278  on 19  degrees of freedom
Residual deviance: 10.331  on 18  degrees of freedom
AIC: NA

Number of Fisher Scoring iterations: 7

> Anova(Eggs.LA.night)
Analysis of Deviance Table (Type II tests)

Response: P_eggs_recovered_night
          LR Chisq Df Pr(>Chisq)
Treatment   27.365  1  1.684e-07 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
>
# DAY:
>
Eggs.LV.day=glm(P_eggs_recovered_day~Treatment,family=quasibinomial(link="logit"),
data=Eggs.LV)
> summary(Eggs.LV.day)

Call:
glm(formula = P_eggs_recovered_day ~ Treatment, family = quasibinomial(link =
"logit"),
    data = Eggs.LV)

Deviance Residuals:
    Min       1Q   Median       3Q      Max
-0.7278  -0.6252   0.1190   0.2154   1.3304

Coefficients:
                  Estimate Std. Error t value Pr(>|t|)
(Intercept)          3.752      1.305   2.875  0.01008 *
TreatmentTreatment  -4.946      1.385  -3.572  0.00218 **
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for quasibinomial family taken to be 0.381604)

    Null deviance: 20.736  on 19  degrees of freedom
Residual deviance:  6.930  on 18  degrees of freedom
AIC: NA

Number of Fisher Scoring iterations: 6

> Anova(Eggs.LV.day)
Analysis of Deviance Table (Type II tests)

Response: P_eggs_recovered_day
          LR Chisq Df Pr(>Chisq)
Treatment   36.178  1  1.801e-09 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
>
>
```

```
# NIGHT:

Eggs.LV.night=glm(P_eggs_recovered_night~Treatment,family=quasibinomial(link="logi
t"),data=Eggs.LV)
> summary(Eggs.LV.night)

Call:
glm(formula = P_eggs_recovered_night ~ Treatment, family = quasibinomial(link =
"logit"),
    data = Eggs.LV)

Deviance Residuals:
    Min       1Q   Median       3Q      Max
-0.5220  -0.5220  -0.1131   0.1514   1.6587

Coefficients:
                  Estimate Std. Error t value Pr(>|t|)
(Intercept)          4.463      1.805   2.473  0.02361 *
TreatmentTreatment   -6.388      1.894  -3.372  0.00339 **
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for quasibinomial family taken to be 0.3669406)

    Null deviance: 23.8486  on 19  degrees of freedom
Residual deviance:  5.2653  on 18  degrees of freedom
AIC: NA

Number of Fisher Scoring iterations: 7

> Anova(Eggs.LV.night)
Analysis of Deviance Table (Type II tests)

Response: P_eggs_recovered_night
          LR Chisq Df Pr(>Chisq)
Treatment   50.644  1  1.107e-12 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
>
# DAY:
>
Eggs.SH.day=glm(P_eggs_recovered_day~Treatment,family=quasibinomial(link="logit"),
data=Eggs.SH)
> summary(Eggs.SH.day)

Call:
glm(formula = P_eggs_recovered_day ~ Treatment, family = quasibinomial(link =
"logit"),
    data = Eggs.SH)

Deviance Residuals:
    Min       1Q   Median       3Q      Max
-1.3961  -0.2840   0.1906   0.8880   0.9734

Coefficients:
                  Estimate Std. Error t value Pr(>|t|)
(Intercept)          3.999      1.714   2.334   0.0314 *
TreatmentTreatment   -3.499      1.777  -1.969   0.0646 .
---
```

```
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for quasibinomial family taken to be 0.5191589)

    Null deviance: 16.532  on 19  degrees of freedom
Residual deviance: 11.703  on 18  degrees of freedom
AIC: NA

Number of Fisher Scoring iterations: 6

> Anova(Eggs.SH.day)
Analysis of Deviance Table (Type II tests)

Response: P_eggs_recovered_day
          LR Chisq Df Pr(>Chisq)
Treatment   9.3011  1    0.00229 **
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
>
# NIGHT:
>
Eggs.SH.night=glm(P_eggs_recovered_night~Treatment,family=quasibinomial(link="logi
t"),data=Eggs.SH)
> summary(Eggs.SH.night)

Call:
glm(formula = P_eggs_recovered_night ~ Treatment, family = quasibinomial(link =
"logit"),
    data = Eggs.SH)

Deviance Residuals:
    Min       1Q   Median       3Q      Max
-1.5525   0.1003   0.1510   0.8440   0.8440

Coefficients:
                  Estimate Std. Error t value Pr(>|t|)
(Intercept)          4.468      2.286   1.955   0.0663 .
TreatmentTreatment  -3.619      2.346  -1.543   0.1403
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for quasibinomial family taken to be 0.5858207)

    Null deviance: 16.078  on 19  degrees of freedom
Residual deviance: 12.245  on 18  degrees of freedom
AIC: NA

Number of Fisher Scoring iterations: 7

> Anova(Eggs.SH.night)
Analysis of Deviance Table (Type II tests)

Response: P_eggs_recovered_night
          LR Chisq Df Pr(>Chisq)
Treatment    6.542  1    0.01054 *
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
>
>
```

```
# DAY:

Eggs.UA.day=glm(P_eggs_recovered_day~Treatment,family=quasibinomial(link="logit"),
data=Eggs.UA)
> summary(Eggs.UA.day)

Call:
glm(formula = P_eggs_recovered_day ~ Treatment, family = quasibinomial(link =
"logit"),
    data = Eggs.UA)

Deviance Residuals:
    Min       1Q   Median       3Q      Max
-1.8886   0.1794   0.1794   0.3513   0.5627

Coefficients:
                  Estimate Std. Error t value Pr(>|t|)
(Intercept)          4.121      1.612   2.557   0.0198 *
TreatmentTreatment   -2.358      1.710  -1.379   0.1848
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for quasibinomial family taken to be 0.4081619)

    Null deviance: 7.2473  on 19  degrees of freedom
Residual deviance: 5.9474  on 18  degrees of freedom
AIC: NA

Number of Fisher Scoring iterations: 7

> Anova(Eggs.UA.day)
Analysis of Deviance Table (Type II tests)

Response: P_eggs_recovered_day
          LR Chisq Df Pr(>Chisq)
Treatment   3.1847  1    0.07433 .
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
>
# NIGHT:
>
Eggs.UA.night=glm(P_eggs_recovered_night~Treatment,family=quasibinomial(link="logi
t"),data=Eggs.UA)
> summary(Eggs.UA.night)

Call:
glm(formula = P_eggs_recovered_night ~ Treatment, family = quasibinomial(link =
"logit"),
    data = Eggs.UA)

Deviance Residuals:
    Min       1Q   Median       3Q      Max
-2.1170   0.1664   0.1664   0.4742   0.4742

Coefficients:
                  Estimate Std. Error t value Pr(>|t|)
(Intercept)          4.273      1.999   2.138   0.0465 *
TreatmentTreatment   -2.144      2.136  -1.004   0.3289
---
```

```
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for quasibinomial family taken to be 0.5417814)

    Null deviance: 7.3858  on 19  degrees of freedom
Residual deviance: 6.5299  on 18  degrees of freedom
AIC: NA

Number of Fisher Scoring iterations: 7

> Anova(Eggs.UA.night)
Analysis of Deviance Table (Type II tests)

Response: P_eggs_recovered_night
          LR Chisq Df Pr(>Chisq)
Treatment   1.5798  1     0.2088
```

***ZEYLANICOBDELLA ARUGAMENSIS* on fish**

```
#Original binomial model on complete dataset (testing for overdispersion):

# DAY:
>
Original.leeches.glm=glm(cbind(Leech_rec_day,Leech_unrec_day)~Shrimp_leech*Treatme
nt_leech,family=binomial(link="logit"),data=Leech2)
> summary(Original.leeches.glm)

Call:
glm(formula = cbind(Leech_rec_day, Leech_unrec_day) ~ Shrimp_leech *
    Treatment_leech, family = binomial(link = "logit"), data = Leech2)

Deviance Residuals:
    Min       1Q   Median       3Q      Max
-4.3620  -0.5938   0.4113   0.4483   5.1905

Coefficients:
                                                        Estimate Std. Error
(Intercept)                                            4.595e+00  1.005e+00
Shrimp_leechLysmata_vittata                            1.452e+01  8.597e+02
Shrimp_leechStenopus_hispidus                         -7.160e-15  1.421e+00
Treatment_leechTreatment                              -5.214e+00  1.027e+00
Shrimp_leechLysmata_vittata:Treatment_leechTreatment  -1.281e+01  8.597e+02
Shrimp_leechStenopus_hispidus:Treatment_leechTreatment -4.269e-01  1.455e+00
                                                       z value Pr(>|z|)
(Intercept)                                              4.572 4.83e-06 ***
Shrimp_leechLysmata_vittata                              0.017    0.987
Shrimp_leechStenopus_hispidus                            0.000    1.000
Treatment_leechTreatment                                -5.079 3.80e-07 ***
Shrimp_leechLysmata_vittata:Treatment_leechTreatment    -0.015    0.988
Shrimp_leechStenopus_hispidus:Treatment_leechTreatment  -0.293    0.769
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for binomial family taken to be 1)

    Null deviance: 511.82  on 59  degrees of freedom
```

```
Residual deviance: 183.06  on 54  degrees of freedom
AIC: 241.43

Number of Fisher Scoring iterations: 16

> Anova(Original.leeches.glm)
Analysis of Deviance Table (Type II tests)

Response: cbind(Leech_rec_day, Leech_unrec_day)
                            LR Chisq Df Pr(>Chisq)
Shrimp_leech                  57.971  2  2.581e-13 ***
Treatment_leech              289.675  1  < 2.2e-16 ***
Shrimp_leech:Treatment_leech   0.361  2     0.8347
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

#Original model using quasibinomial regression and logit link:

# DAY:
>
Original.leeches.Qglm=glm(cbind(Leech_rec_day,Leech_unrec_day)~Shrimp_leech*Treatm
ent_leech,family=quasibinomial(link="logit"),data=Leech2)
> summary(Original.leeches.Qglm)

Call:
glm(formula = cbind(Leech_rec_day, Leech_unrec_day) ~ Shrimp_leech *
    Treatment_leech, family = quasibinomial(link = "logit"),
    data = Leech2)

Deviance Residuals:
    Min       1Q   Median       3Q      Max
-4.3620  -0.5938   0.4113   0.4483   5.1905

Coefficients:
                                                        Estimate Std. Error
(Intercept)                                            4.595e+00  1.768e+00
Shrimp_leechLysmata_vittata                            1.452e+01  1.512e+03
Shrimp_leechStenopus_hispidus                         -7.160e-15  2.500e+00
Treatment_leechTreatment                              -5.214e+00  1.806e+00
Shrimp_leechLysmata_vittata:Treatment_leechTreatment  -1.281e+01  1.512e+03
Shrimp_leechStenopus_hispidus:Treatment_leechTreatment -4.269e-01  2.558e+00
                                                       t value Pr(>|t|)
(Intercept)                                              2.600  0.01201 *
Shrimp_leechLysmata_vittata                              0.010  0.99237
Shrimp_leechStenopus_hispidus                            0.000  1.00000
Treatment_leechTreatment                                -2.888  0.00557 **
Shrimp_leechLysmata_vittata:Treatment_leechTreatment    -0.008  0.99327
Shrimp_leechStenopus_hispidus:Treatment_leechTreatment  -0.167  0.86809
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for quasibinomial family taken to be 3.093173)

    Null deviance: 511.82  on 59  degrees of freedom
Residual deviance: 183.06  on 54  degrees of freedom
AIC: NA

Number of Fisher Scoring iterations: 16
```

```
> Anova(Original.leeches.Qglm)
Analysis of Deviance Table (Type II tests)

Response: cbind(Leech_rec_day, Leech_unrec_day)
                            LR Chisq Df Pr(>Chisq)
Shrimp_leech                  18.741  2  8.518e-05 ***
Treatment_leech               93.650  1  < 2.2e-16 ***
Shrimp_leech:Treatment_leech   0.117  2     0.9433
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

#Pairwise per shrimp species:

# DAY:
>
Leeches.LA.day=glm(Leech_prec_day~Treatment_leech,family=quasibinomial(link="logit
"),data=Leeches.LA)
> summary(Leeches.LA.day)

Call:
glm(formula = Leech_prec_day ~ Treatment_leech, family = quasibinomial(link =
"logit"),
    data = Leeches.LA)

Deviance Residuals:
    Min       1Q   Median       3Q      Max
-0.9282  -0.5479   0.1418   0.1831   1.4490

Coefficients:
                         Estimate Std. Error t value Pr(>|t|)
(Intercept)                 4.595      1.956   2.349   0.0305 *
Treatment_leechTreatment   -5.214      1.999  -2.609   0.0178 *
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for quasibinomial family taken to be 0.3789553)

    Null deviance: 19.2578  on 19  degrees of freedom
Residual deviance:  7.9596  on 18  degrees of freedom
AIC: NA

Number of Fisher Scoring iterations: 7

> Anova(Leeches.LA.day)
Analysis of Deviance Table (Type II tests)

Response: Leech_prec_day
                LR Chisq Df Pr(>Chisq)
Treatment_leech   29.814  1  4.755e-08 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
>
# DAY:
>
Leeches.LV.day=glm(Leech_prec_day~Treatment_leech,family=quasibinomial(link="logit
"),data=Leeches.LV)
> summary(Leeches.LV.day)

Call:
```

```
glm(formula = Leech_prec_day ~ Treatment_leech, family = quasibinomial(link =
"logit"),
    data = Leeches.LV)

Deviance Residuals:
     Min        1Q    Median        3Q       Max
-1.37938  -0.02825   0.00005   0.02962   0.75853

Coefficients:
                          Estimate Std. Error t value Pr(>|t|)
(Intercept)                  20.57    2488.81   0.008    0.993
Treatment_leechTreatment    -19.47    2488.81  -0.008    0.994

(Dispersion parameter for quasibinomial family taken to be 0.197037)

    Null deviance: 7.7585  on 19  degrees of freedom
Residual deviance: 3.9344  on 18  degrees of freedom
AIC: NA

Number of Fisher Scoring iterations: 19

> Anova(Leeches.LV.day)
Analysis of Deviance Table (Type II tests)

Response: Leech_prec_day
                LR Chisq Df Pr(>Chisq)
Treatment_leech   19.408  1  1.056e-05 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
>
# DAY:
>
Leeches.SH.day=glm(Leech_prec_day~Treatment_leech,family=quasibinomial(link="logit
"),data=Leeches.SH)
> summary(Leeches.SH.day)

Call:
glm(formula = Leech_prec_day ~ Treatment_leech, family = quasibinomial(link =
"logit"),
    data = Leeches.SH)

Deviance Residuals:
    Min       1Q   Median       3Q      Max
-0.7760  -0.4015   0.1418   0.1418   1.6414

Coefficients:
                          Estimate Std. Error t value Pr(>|t|)
(Intercept)                  4.595      1.885   2.437  0.02541 *
Treatment_leechTreatment    -5.641      1.933  -2.918  0.00919 **
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for quasibinomial family taken to be 0.3519613)

    Null deviance: 20.2933  on 19  degrees of freedom
Residual deviance:  6.4119  on 18  degrees of freedom
AIC: NA

Number of Fisher Scoring iterations: 7
```

```
> Anova(Leeches.SH.day)
Analysis of Deviance Table (Type II tests)

Response: Leech_prec_day
                LR Chisq Df Pr(>Chisq)
Treatment_leech    39.44  1  3.383e-10 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

### night data for LA only below:

>
Leeches.LA.night=glm(Leech_prec_night~Treatment_leech_night,family=quasibinomial(l
ink="logit"),data=Leech3)
> summary(Leeches.LA.night)

Call:
glm(formula = Leech_prec_night ~ Treatment_leech_night, family =
quasibinomial(link = "logit"),
    data = Leech3)

Deviance Residuals:
    Min       1Q   Median       3Q      Max
-0.7230  -0.3561   0.1811   0.2010   1.2063

Coefficients:
                              Estimate Std. Error t value Pr(>|t|)
(Intercept)                      3.892      1.092   3.563 0.002223 **
Treatment_leech_nightTreatment  -5.100      1.151  -4.430 0.000323 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for quasibinomial family taken to be 0.23389)

    Null deviance: 18.4053  on 19  degrees of freedom
Residual deviance:  4.3143  on 18  degrees of freedom
AIC: NA

Number of Fisher Scoring iterations: 6

> Anova(Leeches.LA.night)
Analysis of Deviance Table (Type II tests)

Response: Leech_prec_night
                     LR Chisq Df Pr(>Chisq)
Treatment_leech_night   60.246  1  8.371e-15 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

#####All analyses present significant treatment effect (shrimp)
```

***ZEYLANICOBDELLA ARUGAMENSIS* cocoons**

```
# Leech cocoons removed by Lysmata vittata only, over 24 hours:
>
Cocoons.glm=glm(Leech_cocoons_prec~Leech_cocoons_treatment,family=quasibinomial(li
nk="logit"),data=Cocoons)
> summary(Cocoons.glm)

Call:
glm(formula = Leech_cocoons_prec ~ Leech_cocoons_treatment, family =
quasibinomial(link = "logit"),
    data = Cocoons)

Deviance Residuals:
     Min        1Q    Median        3Q       Max
-0.30346  -0.30346   0.00002   0.00002   0.78550

Coefficients:
                                Estimate Std. Error t value Pr(>|t|)
(Intercept)                        22.57    4579.85   0.005    0.996
Leech_cocoons_treatmentTreatment   -25.62    4579.85  -0.006    0.996

(Dispersion parameter for quasibinomial family taken to be 0.09029798)

    Null deviance: 25.2386  on 19  degrees of freedom
Residual deviance:  1.2236  on 18  degrees of freedom
AIC: NA

Number of Fisher Scoring iterations: 21

> Anova(Cocoons.glm)
Analysis of Deviance Table (Type II tests)

Response: Leech_cocoons_prec
                       LR Chisq Df Pr(>Chisq)
Leech_cocoons_treatment   265.95  1  < 2.2e-16 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

Appendix 4. Details of the statistical analyses for Chapter 5 using *R*

Effect of *Lysmata vittata* on *N. girellae* infesting *E. lanceolatus*.

```
### Run mixed effects random intercept model with tanks as the random effect and
count data log-transformed:
Model1=lme(log(N_girellaeCH4)~TreatmentCH4*DayCH4,random=~1|TankCH4,data=CH4.data)
> summary(Model1)
Linear mixed-effects model fit by REML
 Data: CH4.data
       AIC      BIC    logLik
  951.3817 984.6714 -467.6909

Random effects:
 Formula: ~1 | TankCH4
        (Intercept)  Residual
StdDev:   0.1917087 0.6237589

Fixed effects: log(N_girellaeCH4) ~ TreatmentCH4 * DayCH4
                             Value  Std.Error  DF   t-value p-value
(Intercept)               6.875135 0.11853900 468  57.99893  0.0000
TreatmentCH4T            -2.114827 0.16763946   6 -12.61533  0.0000
DayCH4Day2               -0.617147 0.09862495 468  -6.25751  0.0000
DayCH4Day3               -0.601230 0.09862495 468  -6.09613  0.0000
TreatmentCH4T:DayCH4Day2  0.750258 0.13947674 468   5.37909  0.0000
TreatmentCH4T:DayCH4Day3  0.325959 0.13947674 468   2.33701  0.0199
 Correlation:
                          (Intr) TrCH4T DCH4D2 DCH4D3 TCH4T:DCH4D2
TreatmentCH4T             -0.707
DayCH4Day2                -0.416  0.294
DayCH4Day3                -0.416  0.294  0.500
TreatmentCH4T:DayCH4Day2  0.294 -0.416 -0.707 -0.354
TreatmentCH4T:DayCH4Day3  0.294 -0.416 -0.354 -0.707  0.500

Standardized Within-Group Residuals:
        Min          Q1         Med          Q3         Max
-3.37876326 -0.56688351 -0.03197522  0.52067114  4.91476087

Number of Observations: 480
Number of Groups: 8
>
>
>
```

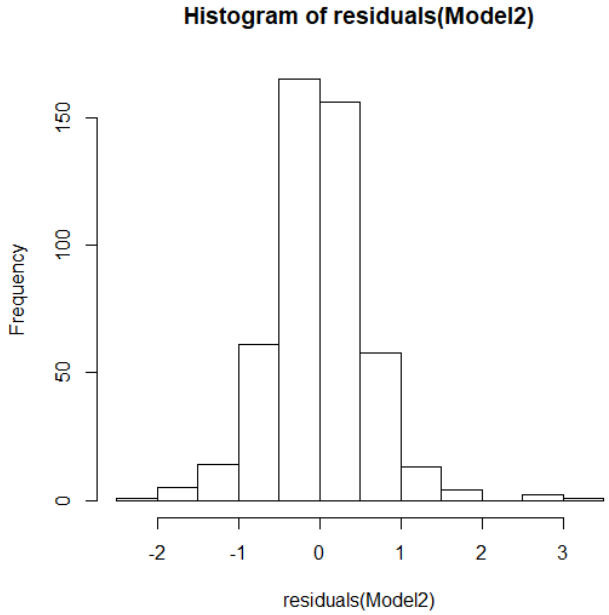
```
Variance function:
 Structure: Different standard deviations per stratum
 Formula: ~1 | TreatmentCH4
 Parameter estimates:
        C         T
1.0000000 0.5600797
Fixed effects: log(N_girellaeCH4) ~ TreatmentCH4 * DayCH4
                             Value Std.Error  DF   t-value p-value
(Intercept)               6.875135 0.1259505 468  54.58601  0.0000
TreatmentCH4T            -2.114827 0.1632197   6 -12.95693  0.0000
DayCH4Day2               -0.617147 0.1217470 468  -5.06909  0.0000
DayCH4Day3               -0.601230 0.1217470 468  -4.93836  0.0000
TreatmentCH4T:DayCH4Day2  0.750258 0.1395419 468   5.37658  0.0000
TreatmentCH4T:DayCH4Day3  0.325959 0.1395419 468   2.33592  0.0199
 Correlation:
                          (Intr) TrCH4T DCH4D2 DCH4D3 TCH4T:DCH4D2
TreatmentCH4T             -0.772
DayCH4Day2                -0.483  0.373
DayCH4Day3                -0.483  0.373  0.500
TreatmentCH4T:DayCH4Day2   0.422 -0.427 -0.872 -0.436
TreatmentCH4T:DayCH4Day3   0.422 -0.427 -0.436 -0.872  0.500

Standardized Within-Group Residuals:
        Min          Q1         Med          Q3         Max
-4.85671049 -0.55309498 -0.04219193  0.65219878  3.96456705

Number of Observations: 480
Number of Groups: 8
>
#### Allow for variance to differ between each treatment-day combination, rather
than just treatments (there are differences in the spread of variance between
days, not just between treatments; see below:)

# See differences in variance between days:
> with(CH4.data, tapply(log(N_girellaeCH4),list(TreatmentCH4,DayCH4),var ))
       Day1      Day2      Day3
C 0.5424765 0.7718764 0.5857066
T 0.3437419 0.1113239 0.1468044
>
# See differences in means between days:
>
> with(CH4.data, tapply(log(N_girellaeCH4),list(TreatmentCH4,DayCH4),mean ))
      Day1     Day2     Day3
C 6.875135 6.257988 6.273905
T 4.760308 4.893419 4.485036

# create vargroup (new variable) whose levels are each treatment-day combination,
and lets each one have its own variance:
> CH4.data$vargp=with(CH4.data,factor(paste(DayCH4,TreatmentCH4)))
> str(CH4.data)
'data.frame':   480 obs. of  6 variables:
 $ LineCH4      : num  1 2 3 4 5 6 7 8 9 10 ...
 $ DayCH4       : Factor w/ 3 levels "Day1","Day2",..: 1 1 1 1 1 1 1 1 1 1 ...
 $ TreatmentCH4 : Factor w/ 2 levels "C","T": 1 1 1 1 1 1 1 1 1 1 ...
 $ N_girellaeCH4: num  559 639 2024 428 1669 ...
 $ TankCH4      : Factor w/ 8 levels "C1","C2","C3",..: 1 1 1 1 1 1 1 1 1 1 ...
 $ vargp        : Factor w/ 6 levels "Day1 C","Day1 T",..: 1 1 1 1 1 1 1 1 1 1 ...
>
# Run third model to include the vargroup:
```

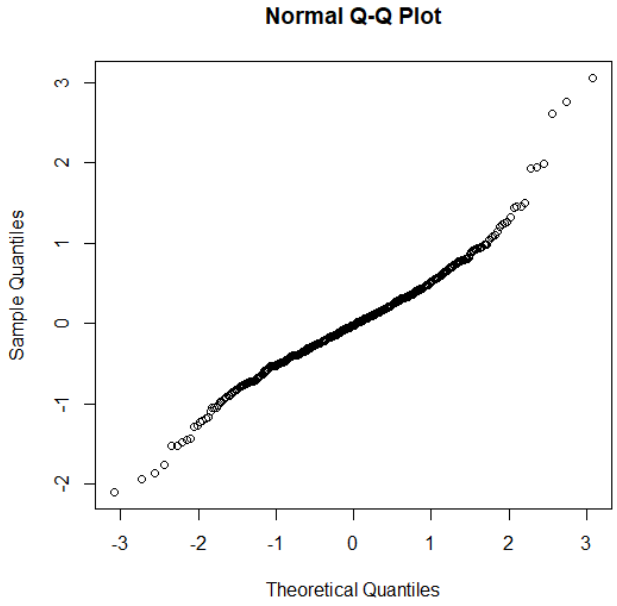
```
Model3=lme(log(N_girellaeCH4)~TreatmentCH4*DayCH4,random=~1|TankCH4,weights=varIde
nt(form=~1|vargp),data=CH4.data)
>
> anova(Model1,Model2,Model3)
       Model df      AIC      BIC    logLik   Test  L.Ratio p-value
Model1     1  8 951.3817 984.6714 -467.6909
Model2     2  9 878.7914 916.2423 -430.3957 1 vs 2 74.59028  <.0001
Model3     3 13 858.3051 912.4008 -416.1526 2 vs 3 28.48630  <.0001
>
# The AIC (lowest) suggests this model is even better (the best).
> summary(Model3)
Linear mixed-effects model fit by REML
 Data: CH4.data
       AIC      BIC    logLik
  858.3051 912.4008 -416.1526

Random effects:
 Formula: ~1 | TankCH4
        (Intercept) Residual
StdDev:   0.1732571 0.699756

Variance function:
 Structure: Different standard deviations per stratum
 Formula: ~1 | vargp
 Parameter estimates:
   Day1 C    Day2 C    Day3 C    Day1 T    Day2 T    Day3 T
1.0000000 1.2214233 1.0679785 0.7989372 0.4482660 0.5573069
Fixed effects: log(N_girellaeCH4) ~ TreatmentCH4 * DayCH4
                            Value Std.Error  DF   t-value p-value
(Intercept)              6.875135 0.1167272 468  58.89917  0.0000
TreatmentCH4T           -2.114827 0.1582296   6 -13.36556  0.0000
DayCH4Day2              -0.617147 0.1234994 468  -4.99717  0.0000
DayCH4Day3              -0.601230 0.1144635 468  -5.25259  0.0000
TreatmentCH4T:DayCH4Day2 0.750258 0.1427896 468   5.25429  0.0000
TreatmentCH4T:DayCH4Day3 0.325959 0.1375129 468   2.37039  0.0182
 Correlation:
                         (Intr) TrCH4T DCH4D2 DCH4D3 TCH4T:DCH4D2
TreatmentCH4T            -0.738
DayCH4Day2               -0.425  0.313
DayCH4Day3               -0.458  0.338  0.433
TreatmentCH4T:DayCH4Day2  0.367 -0.444 -0.865 -0.374
TreatmentCH4T:DayCH4Day3  0.381 -0.461 -0.360 -0.832  0.511

Standardized Within-Group Residuals:
        Min          Q1         Med          Q3         Max
-3.77002063 -0.58999501 -0.01999284  0.67764778  4.07818424

Number of Observations: 480
Number of Groups: 8
>
> anova(Model3)
                   numDF denDF  F-value p-value
(Intercept)            1   468 6858.621  <.0001
TreatmentCH4           1     6  173.357  <.0001
DayCH4                 2   468   31.201  <.0001
TreatmentCH4:DayCH4    2   468   13.870  <.0001
>
# Confidence intervals for Model3 (these are used in table 1 of the manuscript):
> intervals(Model3)
```

```
Approximate 95% confidence intervals

 Fixed effects:
                                lower       est.      upper
(Intercept)                6.64576045  6.8751347  7.1045090
TreatmentCH4T             -2.50200097 -2.1148271 -1.7276531
DayCH4Day2                -0.85982872 -0.6171468 -0.3744649
DayCH4Day3                -0.82615633 -0.6012302 -0.3763041
TreatmentCH4T:DayCH4Day2   0.46966962  0.7502578  1.0308459
TreatmentCH4T:DayCH4Day3   0.05573963  0.3259589  0.5961781
attr(,"label")
[1] "Fixed effects:"

 Random Effects:
  Level: TankCH4
                     lower      est.     upper
sd((Intercept)) 0.08489948 0.1732571 0.3535714

 Variance function:
           lower      est.     upper
Day2 C 0.9809402 1.2214233 1.5208621
Day3 C 0.8582701 1.0679785 1.3289268
Day1 T 0.6400008 0.7989372 0.9973436
Day2 T 0.3585310 0.4482660 0.5604604
Day3 T 0.4461444 0.5573069 0.6961670
attr(,"label")
[1] "Variance function:"

 Within-group standard error:
    lower      est.     upper
0.5986807 0.6997560 0.8178958
> hist(residuals(Model3))
> plot(Model3)
```

The normality of the data is not violated.

Select diagnostic plots





Appendix 5. Additional histological investigation of injuries for Chapter 3

These histological data were not included in Chapter 3 because they did not provide enough resolution over time to either support or reject the results presented in that chapter. As such, they are presented here only as evidence of additional work performed as part of this chapter.

Histology

Immediately following photography and humane euthanasia of all fish, a new sterile scalpel blade was used to excise a dorso-ventral section of skin and underlying musculature from the mid portion of both flanks, through the standardised injury site, and the representative opposite control (uninjured) site of injured fish, or the same area on both left and right flanks of uninjured fish (designated the area of interest, AOI). Each fresh section of tissue was immediately placed into a separate histology cassette, securely closed, labelled, and placed into 10% phosphate-buffered formalin. All tissue samples were allowed to fix for 48 hours before transferral to 70% ethanol and standard automated tissue processing for histology overnight, using an INTESIT-EFTP processor.

After successful tissue processing completion, all labelled cassettes were transferred to a Shandon Histocentre 3 paraffin wax embedding station. Each separate piece of tissue was specifically oriented and embedded side-up in a separate block mould to facilitate a transverse section through all layers of tissue at the cutting face of the block.

Blocks were cut using a MicromHM325 microtome and Feather S35 blades at 4.5 µm. Several sections were cut through the injury and non-injury sites and tissue ribbons floated on a standard warm water bath before selection and delivery to individual charged glass microscope slides. These slides were allowed to air dry at room temperature prior to the completion of, and transfer of the case and a duplicate set to a tissue oven at 60ºC overnight to

remove excess paraffin wax. Thereafter, slides were allowed to cool to room temperature before staining with standard Haematoxylin and Eosin stain and protocol. All slides were covered with a glass coverslip, mounted permanently in DPX (Sigma).

All sections were observed under an Olympus BX53 compound light microscope fitted with DIC (Nomarski) optics. All pathology was scored a rank between 0-8 from normal to infected tissue (Table A5.1; Fig. A5.1).

Table A5.1. Pathology ranking and categories

Rank	Category
0	Normal
1	Disruption of the epidermis; scale loss
2	Epidermal regeneration from sides of wound, not meeting up
3	Epidermal regeneration and alignment of basophilic epidermal cells in scale pockets
4	Inflammatory response
5	Epidermal regeneration from sides of wound, meeting up
6	Epithelial hyperplasia
7	Infection with bacteria or ciliates
8	Inconclusive pathology

Statistical analyses

Pathology rankings for the tissue sections were analysed using mixed effects random intercept models using the package 'lme4'(Bates *et al*. 2015) in the R (Version 3.4.0; R Development Core Team 2017). Data passed diagnostic scrutiny for normality (see plots presented at the end of this appendix). I analysed the *frequency* of ranks (the response variable) as a function of the fixed effects, *treatment* (two levels: *with_shrimp; without_shrimp*), rank *category* (eight levels: 0-8), day (three levels: 2, 4, 6), and the random effect of fish *side*, and interactions between them: *treatment* x *category* x (1|*side*) x *day*. To determine the significance of the *treatment* (the presence or absence of shrimp) on pathology, two models representing the hypothesis that the presence of shrimp influences pathology, were constructed; one including, and one excluding

treatment (presented at the end of this appendix). Both models were compared using the anova() function.

Results

There was no difference in the pathology in fish cohabited with, or kept without shrimp: X^2 (56, n = 108) = 11.12, p =0.997). However, only fish that were not cohabited with shrimp were noted with any secondary infection with either bacteria, and/or ciliates (see Fig. A5.1).

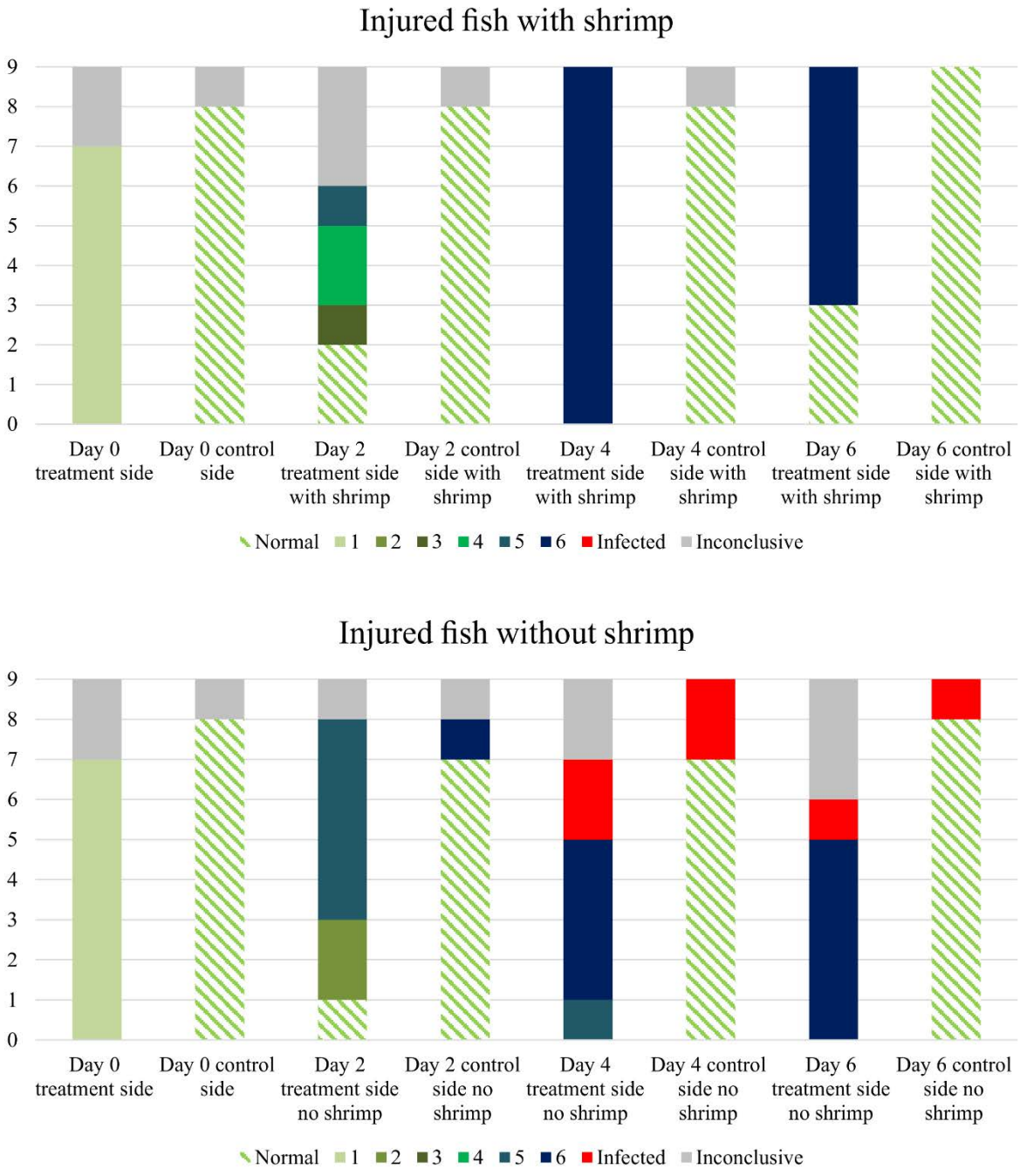


Fig. A5.1. Pathology recorded in fish cohabited with and without shrimp.

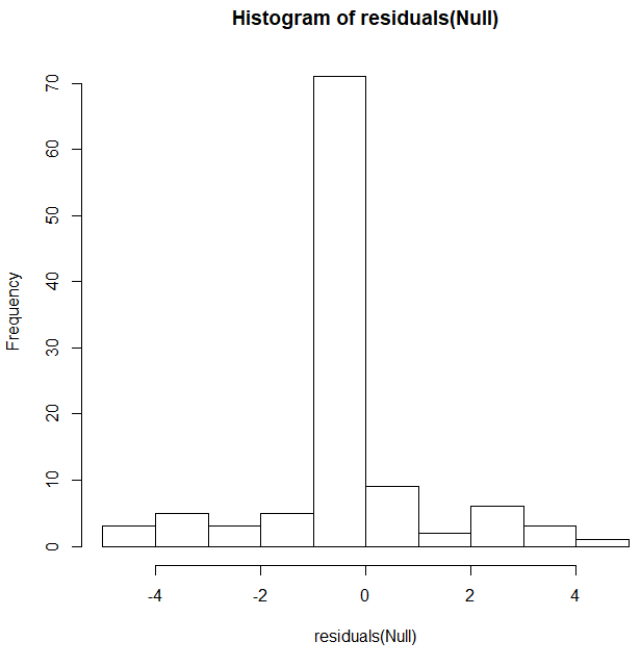
Discussion

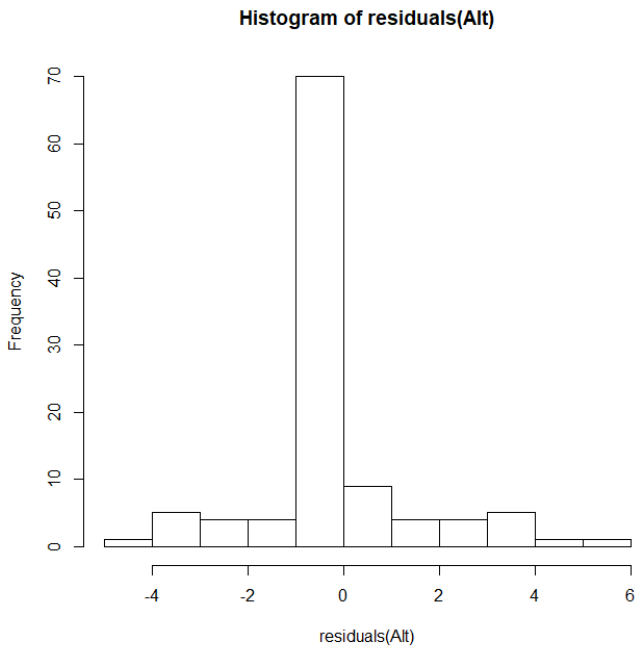
The results of the histological interpretation of the effect of the presence or absence of shrimp on skin pathology, were inconclusive. This may simply reflect the fact of the superficial nature of the standardised injury, and therefore the rapid and relatively uneventful epithelial tissue response. Although few fish that were not cohabited with shrimp developed a bacterial and/or ciliate infection, these few individuals were not significant to influence the statistical model, and therefore infection should be considered incidental.

Statistical analyses and diagnostic plots

```
> Hlm2=lmer(FrequencyH~TreatmentH*CategoryH*(1|SideH)*DayH,data=Histo)
> summary(Hlm2)
Linear mixed model fit by REML ['lmerMod']
Formula: FrequencyH ~ TreatmentH * CategoryH * (1 | SideH) * DayH
   Data: Histo

REML criterion at convergence: 273.2

Scaled residuals:
   Min     1Q Median     3Q    Max
-2.096  0.000  0.000  0.000  2.096

Random effects:
 Groups   Name        Variance  Std.Dev.
 SideH    (Intercept) 6.640e-06 0.002577
 Residual             4.611e+00 2.147350
Number of obs: 108, groups:  SideH, 2

Fixed effects:
                                                 Estimate Std. Error t value
(Intercept)                                    -6.667e-15  1.518e+00   0.000
TreatmentHWith shrimp                          -2.484e-15  2.147e+00   0.000
CategoryH2                                      1.000e+00  2.147e+00   0.466
CategoryH3                                      7.949e-15  2.147e+00   0.000
CategoryH4                                      7.200e-15  2.147e+00   0.000
CategoryH5                                      2.500e+00  2.147e+00   1.164
CategoryH6                                      5.000e-01  2.147e+00   0.233
CategoryHInconclusive                           1.000e+00  2.147e+00   0.466
CategoryHInfected                               7.850e-15  2.147e+00   0.000
CategoryHNormal                                 4.000e+00  2.147e+00   1.863
DayH4                                           5.286e-15  2.147e+00   0.000
DayH6                                           9.201e-15  2.147e+00   0.000
TreatmentHWith shrimp:CategoryH2               -1.000e+00  3.037e+00  -0.329
TreatmentHWith shrimp:CategoryH3                5.000e-01  3.037e+00   0.165
TreatmentHWith shrimp:CategoryH4                1.000e+00  3.037e+00   0.329
TreatmentHWith shrimp:CategoryH5               -2.000e+00  3.037e+00  -0.659
TreatmentHWith shrimp:CategoryH6               -5.000e-01  3.037e+00  -0.165
```

```
TreatmentHWith shrimp:CategoryHInconclusive        1.000e+00  3.037e+00   0.329
TreatmentHWith shrimp:CategoryHInfected            1.413e-15  3.037e+00   0.000
TreatmentHWith shrimp:CategoryHNormal              1.000e+00  3.037e+00   0.329
TreatmentHWith shrimp:DayH4                        5.685e-15  3.037e+00   0.000
TreatmentHWith shrimp:DayH6                       -1.306e-15  3.037e+00   0.000
CategoryH2:DayH4                                  -1.000e+00  3.037e+00  -0.329
CategoryH3:DayH4                                  -6.778e-15  3.037e+00   0.000
CategoryH4:DayH4                                  -6.205e-15  3.037e+00   0.000
CategoryH5:DayH4                                  -2.000e+00  3.037e+00  -0.659
CategoryH6:DayH4                                   1.500e+00  3.037e+00   0.494
CategoryHInconclusive:DayH4                       -1.099e-15  3.037e+00   0.000
CategoryHInfected:DayH4                            2.000e+00  3.037e+00   0.659
CategoryHNormal:DayH4                             -5.000e-01  3.037e+00  -0.165
CategoryH2:DayH6                                  -1.000e+00  3.037e+00  -0.329
CategoryH3:DayH6                                  -1.080e-14  3.037e+00   0.000
CategoryH4:DayH6                                  -1.026e-14  3.037e+00   0.000
CategoryH5:DayH6                                  -2.500e+00  3.037e+00  -0.823
CategoryH6:DayH6                                   2.000e+00  3.037e+00   0.659
CategoryHInconclusive:DayH6                        5.000e-01  3.037e+00   0.165
CategoryHInfected:DayH6                            1.000e+00  3.037e+00   0.329
CategoryHNormal:DayH6                             -2.076e-14  3.037e+00   0.000
TreatmentHWith shrimp:CategoryH2:DayH4             1.000e+00  4.295e+00   0.233
TreatmentHWith shrimp:CategoryH3:DayH4            -5.000e-01  4.295e+00  -0.116
TreatmentHWith shrimp:CategoryH4:DayH4            -1.000e+00  4.295e+00  -0.233
TreatmentHWith shrimp:CategoryH5:DayH4             1.500e+00  4.295e+00   0.349
TreatmentHWith shrimp:CategoryH6:DayH4             3.000e+00  4.295e+00   0.698
TreatmentHWith shrimp:CategoryHInconclusive:DayH4 -1.500e+00  4.295e+00  -0.349
TreatmentHWith shrimp:CategoryHInfected:DayH4     -2.000e+00  4.295e+00  -0.466
TreatmentHWith shrimp:CategoryHNormal:DayH4       -5.000e-01  4.295e+00  -0.116
TreatmentHWith shrimp:CategoryH2:DayH6             1.000e+00  4.295e+00   0.233
TreatmentHWith shrimp:CategoryH3:DayH6            -5.000e-01  4.295e+00  -0.116
TreatmentHWith shrimp:CategoryH4:DayH6            -1.000e+00  4.295e+00  -0.233
TreatmentHWith shrimp:CategoryH5:DayH6             2.000e+00  4.295e+00   0.466
TreatmentHWith shrimp:CategoryH6:DayH6             1.000e+00  4.295e+00   0.233
TreatmentHWith shrimp:CategoryHInconclusive:DayH6 -2.500e+00  4.295e+00  -0.582
TreatmentHWith shrimp:CategoryHInfected:DayH6     -1.000e+00  4.295e+00  -0.233
TreatmentHWith shrimp:CategoryHNormal:DayH6        1.000e+00  4.295e+00   0.233

Correlation matrix not shown by default, as p = 54 > 12.
Use print(x, correlation=TRUE) or vcov(x) if you need it

> Null=lmer(FrequencyH~TreatmentH*CategoryH*(1|SideH)*DayH,data=Histo)

> Alt=lmer(FrequencyH~CategoryH*(1|SideH)*DayH,data=Histo)

> anova(Null,Alt)
refitting model(s) with ML (instead of REML)
Data: Histo
Models:
Alt: FrequencyH ~ CategoryH * (1 | SideH) * DayH
Null: FrequencyH ~ TreatmentH * CategoryH * (1 | SideH) * DayH
     Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
Alt  29 465.82 543.61 -203.91   407.82
Null 56 508.71 658.90 -198.35   396.71 11.118     27      0.997
```


Histogram of residuals(Null)
Frequency
0
10
20
30
40
50
60
70
-4
-2
0
2
4
residuals(Null)


Histogram of residuals(Alt)
Frequency
0
10
20
30
40
50
60
70
-4
-2
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Appendix 6

UV reflectance in the cleaner shrimp *Stenopus hispidus*

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This appendix represents an observation that may or may not be of importance between cleaner shrimp and clients, and which required further verification using several endangered species (sea turtles), which was ethically infeasible.

Abstract

Cleaning symbiosis is an interspecific mutualism proceeded by communication. This communication includes attraction signalling by many cleaners that service clients diurnally, while tactile stimulation is the primary method of communication in low light conditions, or nocturnally. Cleaner fishes which reflect the blue spectrum, including the longer wavelengths, and long-wave ultraviolet, are highly conspicuous to a range of reef fishes' visual systems. Cleaner shrimp have never been tested for their ability to reflect long-wave ultraviolet, and therefore it is unknown whether they too reflect wavelengths that may be potentially conspicuous to receptive clients. I demonstrate for the first time that a cleaner shrimp, *Stenopus hispidus* reflects long-wave ultraviolet, but that other species, including the conspicuous diurnal cleaner *Lysmata amboinensis*, do not. I discuss the possibility that *S. hispidus* may use

long-wave ultraviolet reflectance diurnally to attract a specific clientele sensitive to ultraviolet, including sea turtles.

Introduction

Many cleaner shrimp and fishes are visually conspicuous, displaying bright colouration and prominent contrasting markings thought to play a role in attracting clients to be cleaned. These markings and colours have been investigated for cleaner fishes off shallow inshore reefs, but have never been formally investigated for cleaner shrimp. Two somewhat overlapping hypotheses currently attempt to explain attraction or status signalling by cleaner fishes to client fishes: the guild mark hypothesis (Eibl-Eibesfeldt 1955), and the blue colour guild hypothesis (see Stummer *et al.* 2004; Cheney *et al.* 2009; Chapter 1).

The similarity in markings displayed by different cleaner fishes from different geographic locations prompted the proposal of the cleaner guild mark hypothesis in the mid-1950s; that similar markings had evolved in different fish species to specifically advertise their cleaner status (Eibl-Eibesfeldt 1955; Potts 1973). The guild mark hypothesis was historically supported by the presence of contrasting dark longitudinal stripes, or a dark tail spot or bar on cleaner fishes (Eibl-Eibesfeldt 1955; Potts 1968, 1973; Ayling and Grace 1971). However, when analysed by Côté (2000), only dedicated cleaners (see definitions in Vaughan *et al.* 2016; Chapter 2) offered significant support for this hypothesis. These analyses compared intra-generically for dedicated cleaners (*Elacatinus* spp.), yet inter-generically for facultative cleaners (see Appendix 1 of Côté 2000), and were therefore confounded.

Cheney *et al.* (2009) stated that both colour and pattern were important in cleaner fish signals. In their analyses, all cleaner fishes were likely to display a black lateral stripe against contrasting adjacent 'Blue/Red' (peak reflectance at 450–500 nm; step in reflectance at 650–700 nm), 'UV/Blue' (<400 nm, and >400 nm), 'Yellow' (step at ~500 nm), and/or

'UV/Yellow' (step at ~500 nm, and additional peak at <400 nm), or the converse, compared to non-cleaners (Cheney *et al.* 2009). These authors concluded that variations of blue (i.e. 'UV/Blue', 'Blue/Red') offered significant long distance conspicuousness to a range of known client fish visual systems, including clients sensitive to ultraviolet (Cheney *et al*. 2009).

Contrasting patterns in cleaner shrimp include red against white, not black as in cleaner fishes. Many cleaner shrimp species possess white antennal or antennual flagella (Karplus 2014), white appendages, or a white mid-dorsal stripe (Wicksten 2009). The white colour of these structures was considered a necessary feature for a shrimp to be considered a cleaner (Wicksten 2009), which is also partly supported by Bruce (1976) who mentioned that non-cleaner shrimp rarely possess this white colouration. However, not all cleaner shrimp have white antennal or antennual flagella (e.g. *Stenopus tenuirostris* de Mann, 1888, and *Urocaridella antonbruunii* (Bruce, 1967); Calado 2008), which might indicate that the colour white serves more for visual recognition during the day. Karplus (2014) emphasised the potential lucidity of red and white alternating colouration in various cleaner shrimp and suggested that a study of the sensitivity of fishes to this colour pattern should be investigated. However, longer wavelengths of visible light such as red attenuate first with depth in seawater, thus red appears dark or black with increased depth, and may function more in contrast, as dark guild marks do in cleaner fishes, than as a colour signal. However, the spectral reflectance of the white colouration of these shrimp has not been investigated. Although the 'UV/Blue' and 'Blue/Red' categories supporting the blue colour guild hypothesis in cleaner fishes both include blue wavelengths visible to the naked eye (>400 nm), cleaner shrimp that reflect long-wave ultraviolet (<400 nm) from visibly white body markings would still be conspicuous to clients sensitive to 'UV/Blue', and would therefore support the blue colour guild hypothesis, at least partially. I aimed to test this using four common tropical cleaner shrimp species; two known diurnal cleaners with visibly conspicuous white-red alternating markings (*Lysmata*

amboinensis (de Man, 1888), and *Stenopus hispidus* (Olivier, 1811)), and two nocturnal cleaners with less generally conspicuous contrasting colour patterns (*Lysmata vittata* (Stimpson, 1860), and *Urocaridella antonbruunii* (Bruce, 1967)). Of these, *L. amboinensis* and *U. antonbruunii* are dedicated cleaners, while *L. vittata* and *S. hispidus* are facultative cleaners (Vaughan *et al*. 2016; Chapter 2).

Methods

The use of cleaner shrimp was approved under the James Cook University animal ethics permit number A2260, granted to DBV. Ten wild-caught adult individuals of each cleaner shrimp species were purchased from an approved Australian commercial supplier (Cairns Marine), and kept individually in 3 L polypropylene aquaria connected to a recirculating marine life-support system. Cleaner shrimp moult relatively frequently as they grow (Wong and Michiels 2011), and stop feeding prior to moulting. To ensure shrimp were not about to moult, and therefore that the carapace to be photographed was not old and about to be shed, all shrimp were confirmed to be actively feeding prior to photography. Individual shrimp were placed into a 3 L polyethylene aquarium painted black with non-reflective paint for long-wave ultraviolet photography. Shrimp were anaesthetised with clove oil mixed at a ratio of 1:10 clove oil to ethanol, and using 0.2 ml of this solution per litre of fresh, filtered seawater, for the duration of photography to prevent movement. All long-wave ultraviolet photographs were taken with an XNite Nikon D3300 UV-only enabled SLR digital camera fitted with an XNite 330C UV-pass only filter (250 nm–400 nm; see Fig. A6.1 for spectral performance). The camera was mounted directly above the tank with a tripod, and photography was done inside a darkroom under long-wave ultraviolet irradiation using a 25 W (GL-UVB22) long-wave ultraviolet fluorescent light source.

All photographs were processed using FIJI image analysis software. Each RGB image was converted to the C.I.E. (Commission internationale de l'éclairage) *L*a*b** colour space (*CIELAB*) and separated into its three channels using the menu functions *Image_Stacks*, and *Stacks_to_images*. The *a** channel (red–green) was selected for image analysis because the false-colour RGB image produced by the camera, labelled UV reflectance (GL-UVB22) with pink/red pixels (Fig. A6.2a). Image segmentation was performed on the *a** channel images (Fig. A6.2b) using thresholding. The upper threshold reference value was considered the upper UV wavelength limit of the XNite 330C filter; 400 nm. *Dark background* was selected as the default, and background pixels were set to *NaN*, allowing a full threshold view of all labelled pixels in default red against a black background (Fig. A6.2c).

Results and discussion

Only *S. hispidus* reflected long-wave ultraviolet light, corresponding with the white banded patterns of the carapace and chelipeds, and antennae (Fig. A6.2). My results corresponded with an unpublished spectral analysis of *S. hispidus* by Karen Cheney and Justin Marshall (Fig. A6.3) which estimated this UV spectral reflectance between 300 nm–400 nm, with an increase in percentage reflectance towards the longer wavelengths to a maximum of ~40%. The prominent white longitudinal stripe of *L. amboinensis* did not reflect long-wave ultraviolet light (Fig. A6.4), and both *Lysmata* species and *U. antonbruunii* were all but invisible to long-wave ultraviolet photography (Figs. A6.5, A6.6). Currently, a general paucity of knowledge exists for the diurnal or nocturnal cleaning preferences of different cleaner shrimp species, but the presence of long-wave ultraviolet reflectance or reflectance in the blue spectrum may assist in determining which cleaner shrimp species may function actively during the day. My results for *S. hispidus* may partially support the blue colour guild hypothesis, and therefore that this shrimp signals diurnally to clients that are sensitive to long-wave ultraviolet.

Stenopus hispidus is generally considered a poor performer of fish parasite reduction in the literature, and has been questioned as a cleaner shrimp due to this specific lack of perceived performance (see Bunkley-Williams and Williams 1998; McCammon *et al*. 2010). However, this shrimp species has specifically been observed cleaning juvenile Hawksbill turtles (*Eretmochelys imbricata* Linnaeus, 1766) diurnally of epibionts and possibly dead skin in shallow waters (Sazima *et al*. 2004a). Interactions between *E. imbricata* and *S. hispidus* were not merely incidental, but reflected repeated, determined interactions by resident turtles to the study area (Sazima *et al*. 2004a). In addition to the turtles, three fish client species were also observed being cleaned at the same cleaning stations by *S. hispidus*, *Acanthurus chirurgus* (Bloch, 1787), *Haemulon parra* (Desmarest, 1823), and *Sparisoma axillare* (Steindachner, 1878).

Sea turtles including *Chelonia mydas* (Linnaeus, 1758), *Caretta caretta* (Linnaeus, 1758), *Dermochelys coriacea* (Vandelli, 1761), and *E. imbricata* are sensitive to ultraviolet wavelengths (Fritsches and Warrant 2013; Wang *et al*. 2013), and the former two fish species are members of families known to have visual UV spectral sensitivity (Siebeck and Marshall 2001). *Stenopus hispidus* may therefore service a particular clientele niche, which includes turtles, but which requires further focused investigation. Turtles are also known to seek out cleaning interactions with cleaner fishes (Booth and Peters 1972; Smith 1988; Losey *et al.* 1994; Sazima *et al*. 2004b).

Lysmata amboinensis and *S. hispidus* are both known to be active diurnally as well as nocturnally (Collette and Talbot 1972; Corredor 1978; Jonassen 1987; Sazima *et al*. 2004a; Militz and Hutson 2015; Esaka *et al*. 2016; Chapter 4). However, the evidence that *L. amboinensis* does not reflect long-wave ultraviolet suggests that its diurnally visible prominent white markings may attract a different diurnal clientele to *S. hispidus*, in particular, those without a UV-sensitive visual system, and may add support to explain the cleaning discordance

phenomenon discussed by Titus *et al*. (2015). This cleaning discordance was initially identified between cleaner shrimp and cleaner fishes, evident by the lack of competition between shrimp and fish cleaners. Titus *et al.* (2015) suggested that one explanation might be that these different cleaners might offer different cleaning services. This lack of competition may also exist between cleaner shrimp species that occupy the same reef systems, either as a function of shrimp diurnal or nocturnal activity, or by advertising diurnally to clients of different visual systems. In particular diurnal cleaner shrimp colouration without blue or UV reflectance may serve as a visual signal to resident client species in immediate vicinity of the cleaner, while those with UV reflectance may attract clients over longer distances, at least in shallow waters.

The lack of any functional long-wave ultraviolet reflectance in *L. vittata* and *U. antonbruunii* together with the lack of lucid visual markings, supports the consideration of Marin *et al*. (2012) and recent observations by Bonaldo *et al.* (2015), and Bros and Fransen (2018) that these shrimp are primarily nocturnal cleaners, and therefore that visual signalling in these species is likely irrelevant. Communication, either visually, or through tactile stimulation, is considered a prerequisite of cleaning symbiosis (Vaughan *et al*. 2016; Chapter 2). Tactile stimulation is likely the primary means of communicating cleaning intent to a prospective client nocturnally, or under low light levels, and was recently supported by observations of deep water cleaner shrimp made by submersible at 280–320 m (Moura *et al.* 2018).

Further work is necessary to explore the spectral reflectance of different cleaner shrimp, particularly those which are known to clean diurnally. I was restricted to four locally available species, but other species, such as *Ancylomenes pedersoni* possess visibly blue or violet markings which may further add support to the blue colour guild hypothesis in some diurnal cleaner shrimp.

Acknowledgements

I am grateful for the assistance given by Pauline Narvaez during the long-wave ultraviolet photography of cleaner shrimp. Permission to use the UV spectral data for *S. hispidus* was granted by Karen Cheney and Justin Marshall, The University of Queensland. Permission to use Fig. A6.1 was granted by Dan Llewellyn, LDP LLC, www.MaxMax.com. DBV was funded through an International Post-graduate Research Scholarship by James Cook University, Townsville, Queensland, Australia.

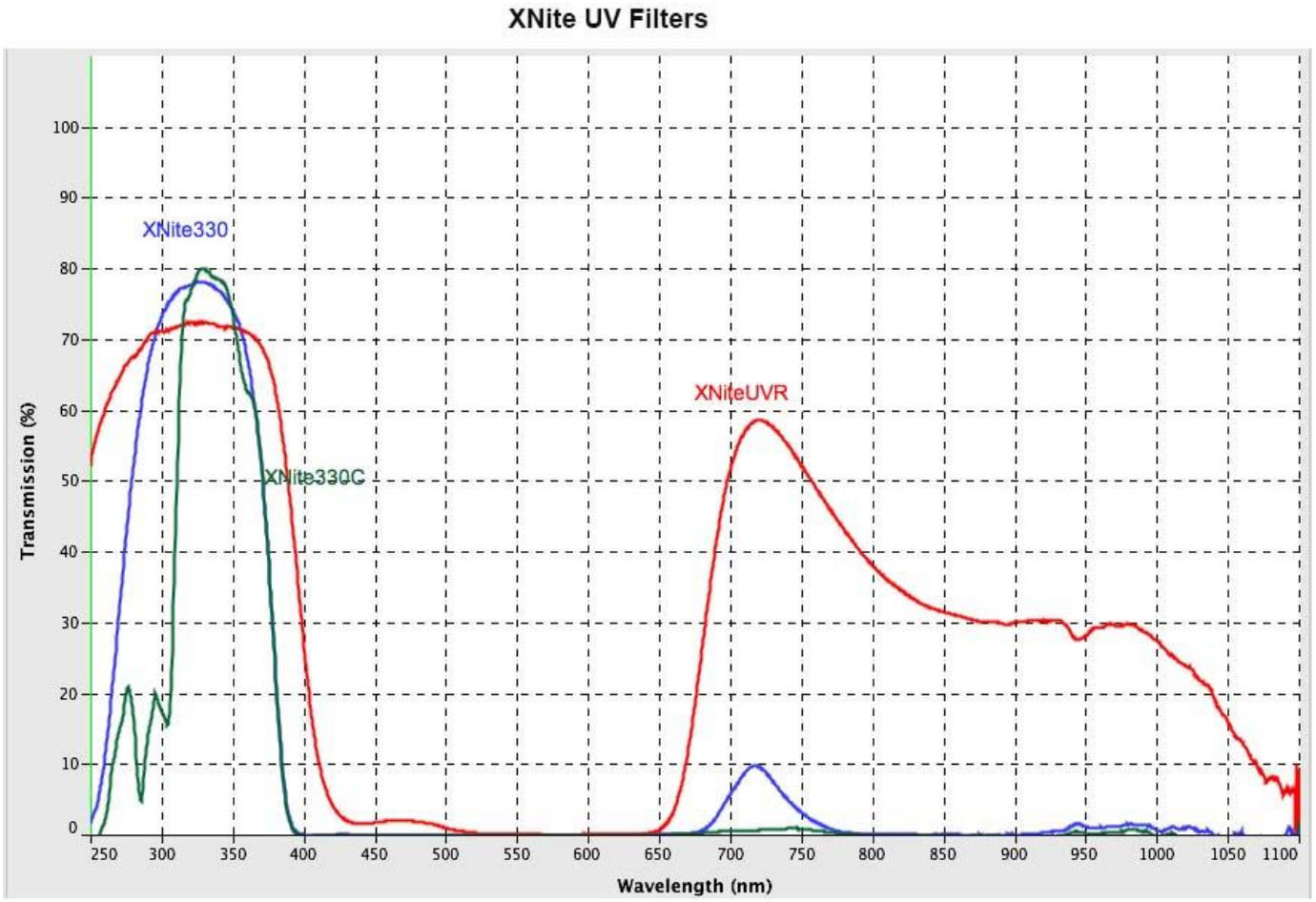

Fig. A6.1. Spectral performance for the XNite 330C UV-pass camera filter (green); supplied by and used with permission from Dan Llewellyn, LDP LLC www.MaxMax.com

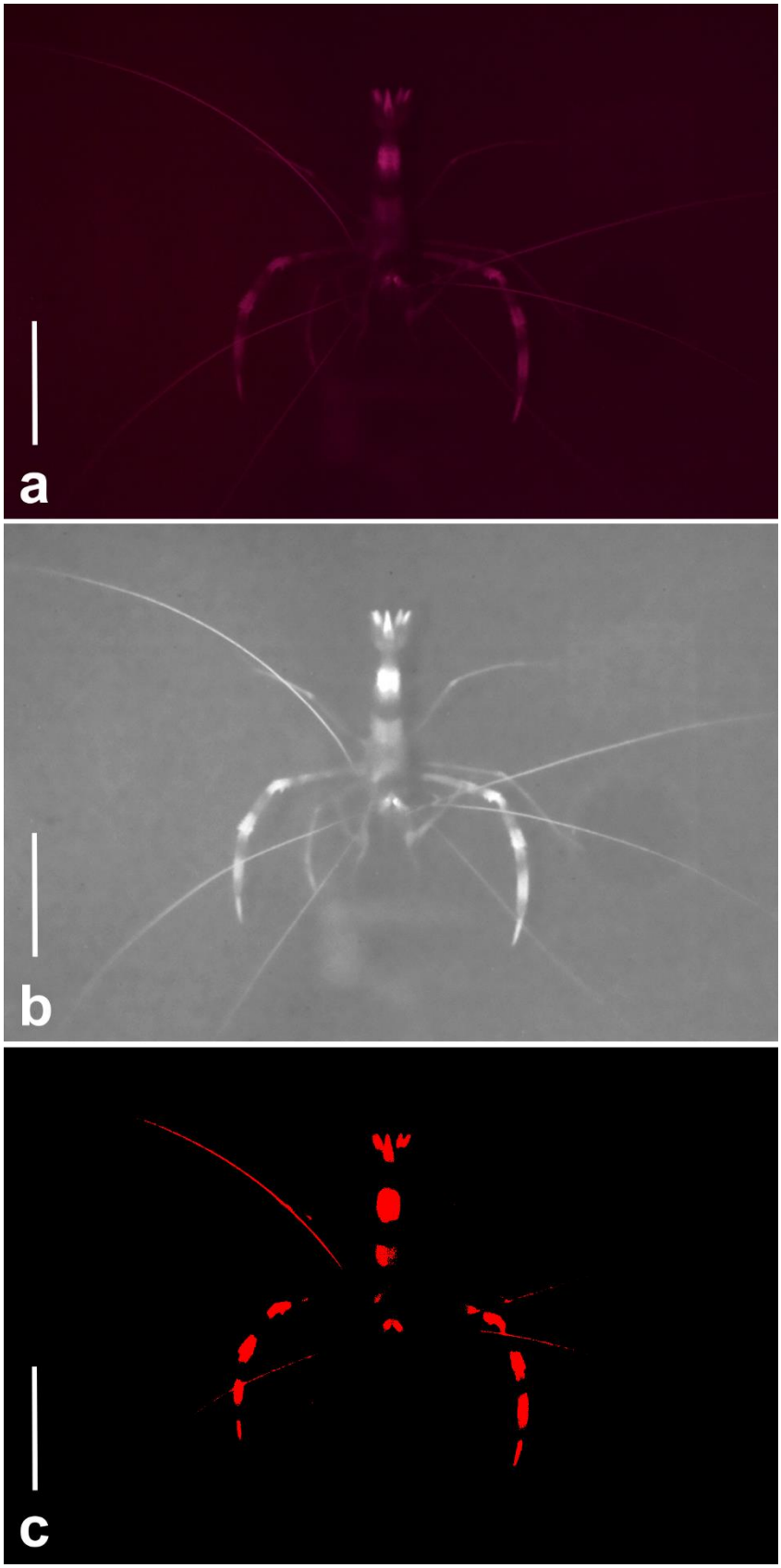

Fig. A6.2. *Stenopus hispidus* photographed under long-wave ultraviolet irradiation; a. raw false-colour RGB image; b. the *a** channel of the C.I.E. *L*a*b** colour space-converted RGB image; c. threshold view of ultraviolet reflective body sections using labelled pixels in red. Scale bars = 25 mm.

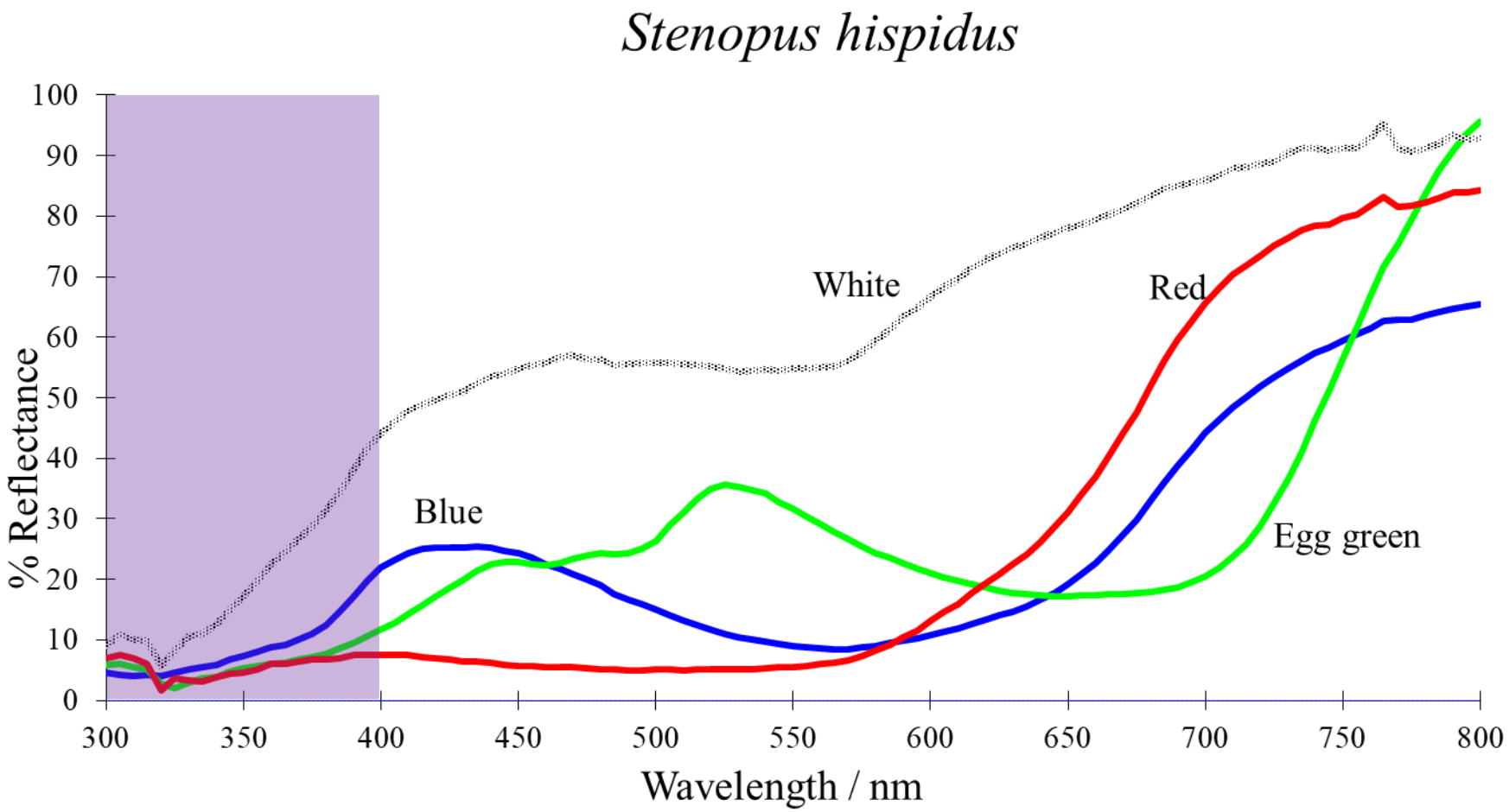

Fig. A6.3. *Stenopus hispidus* UV spectral reflectance from white, red, and blue body colouration, and the green of the eggs, courtesy of Karen Cheney and Justin Marshall.

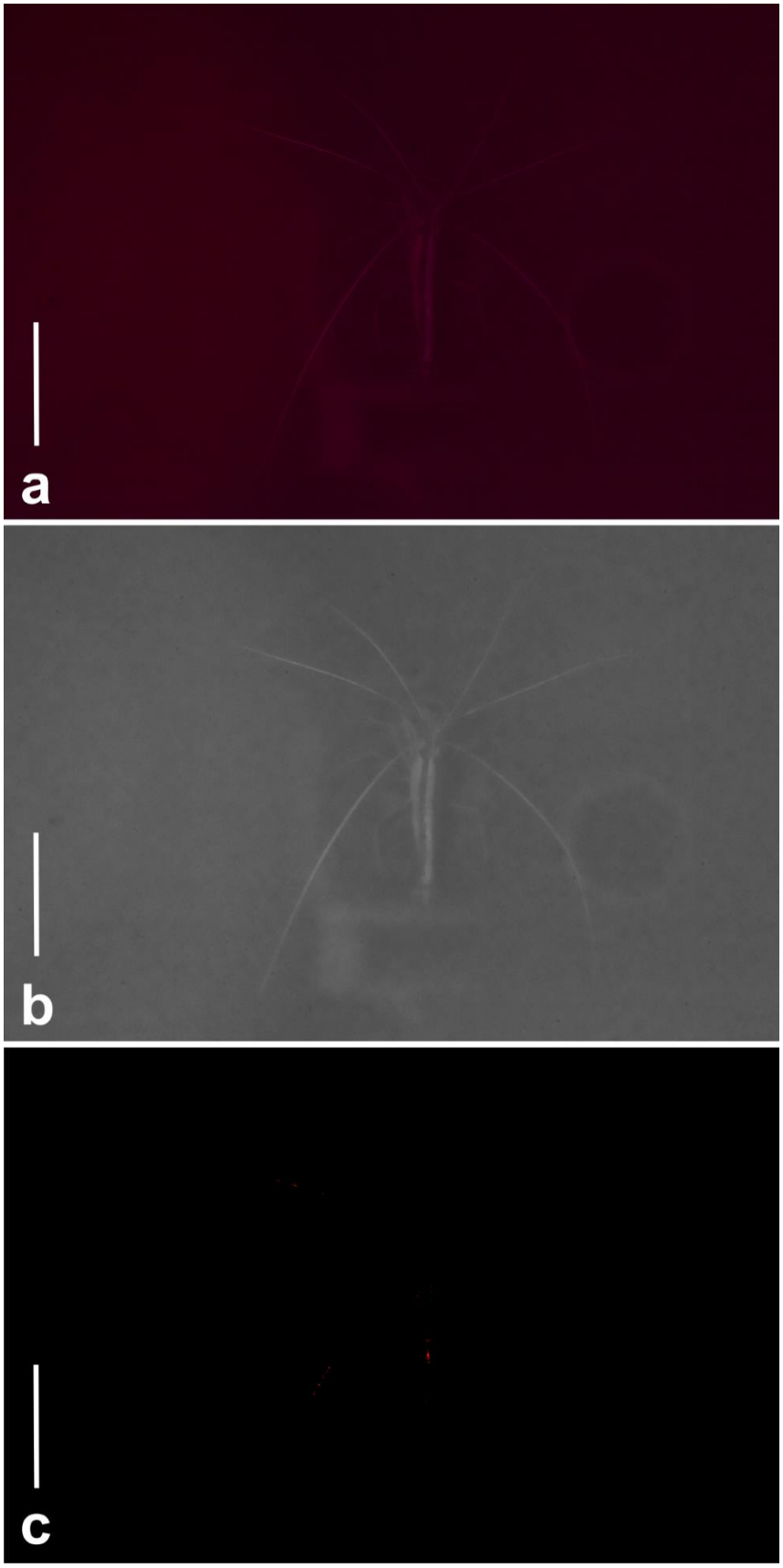

Fig. A6.4. *Lysmata amboinensis;* a. raw false-colour RGB image; b. the *a** channel; c. threshold view. Scale bars = 25 mm.

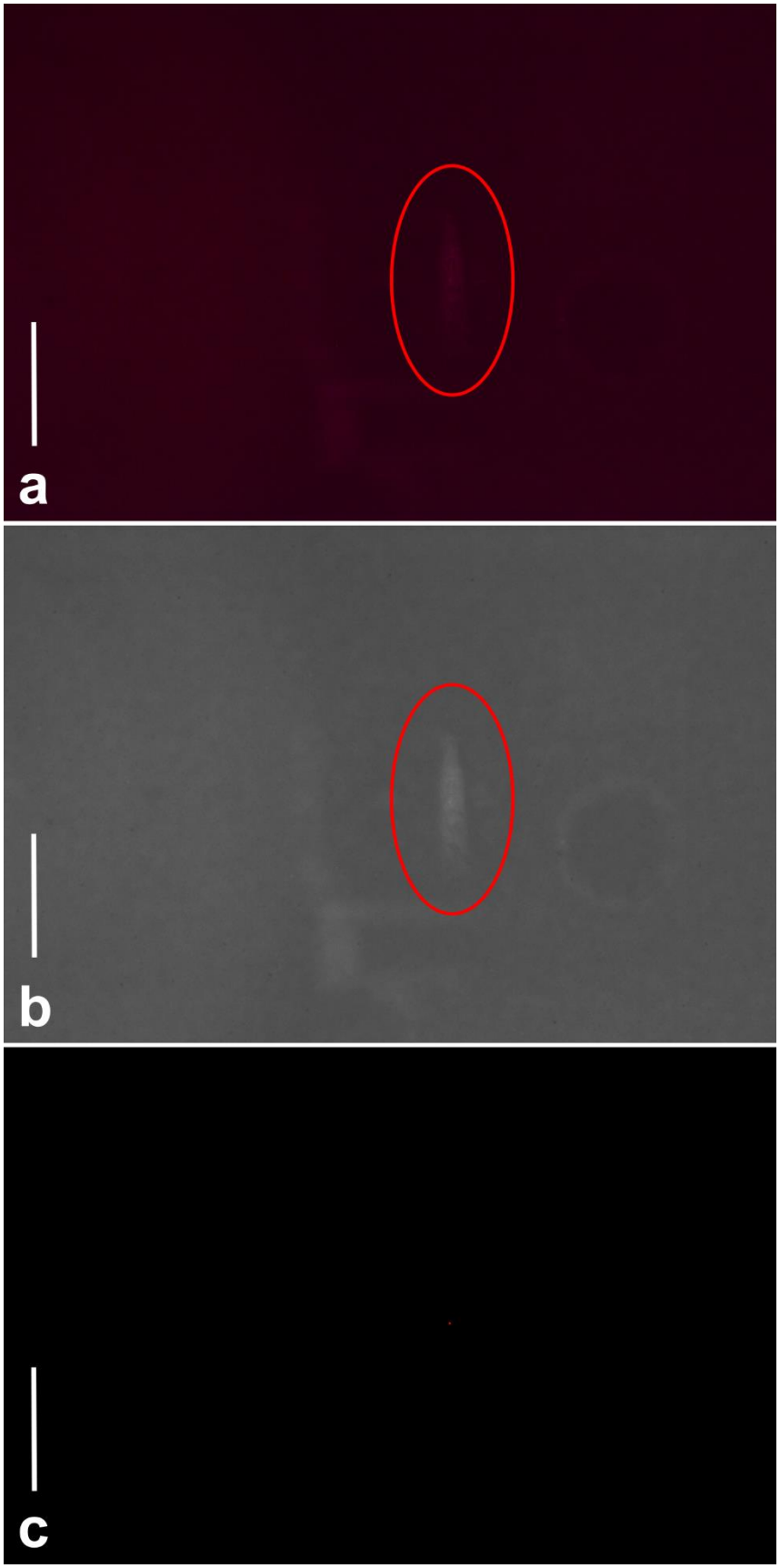

Fig. A6.5. *Lysmata vittata;* a. raw false-colour RGB image; b. the *a** channel; c. threshold view. Scale bars = 25 mm.

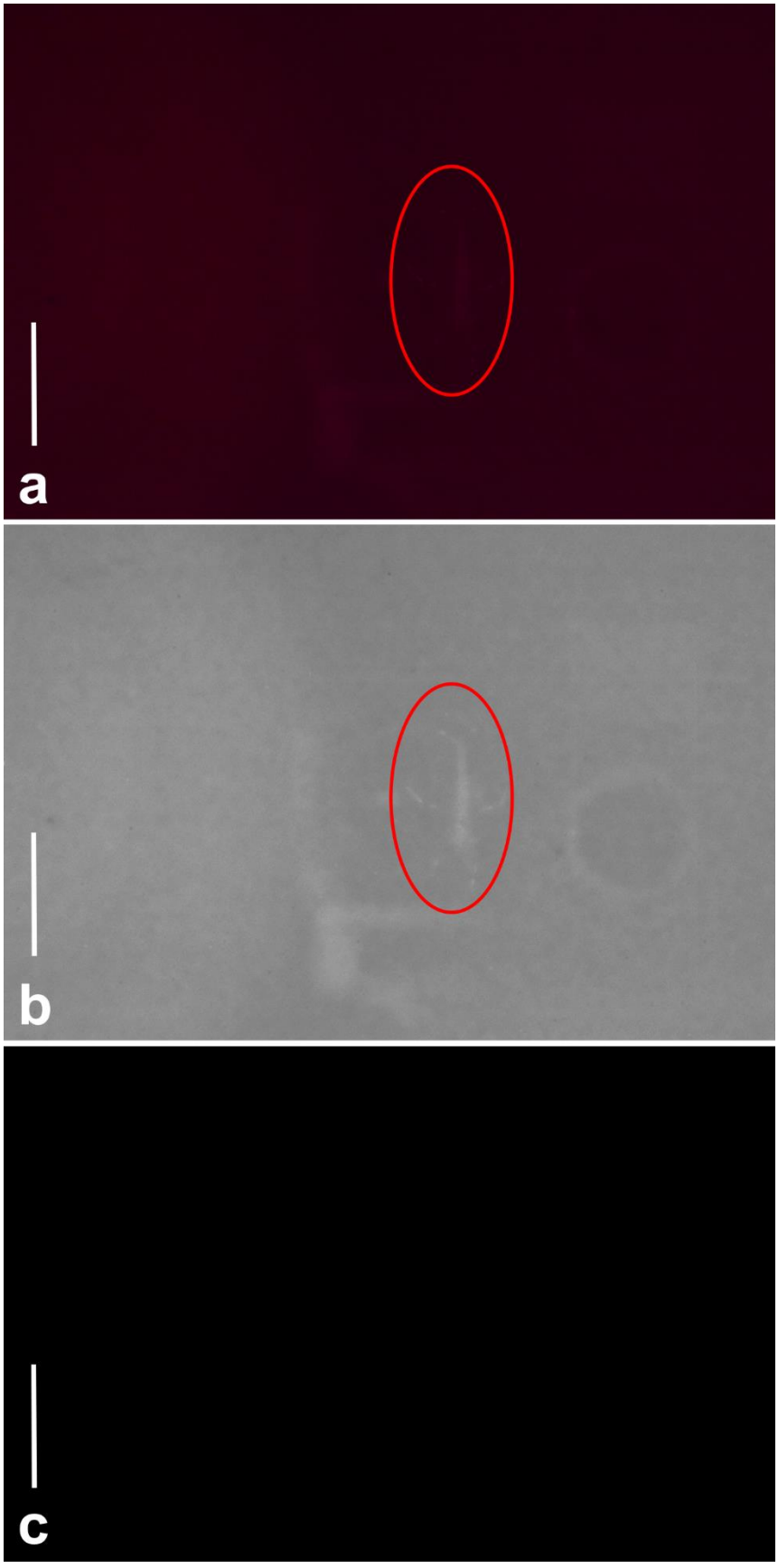

Fig. A6.6. *Urocaridella antonbruunii;* a. raw false-colour RGB image; b. the *a** channel; c. threshold view. Scale bars = 25 mm.

Appendix 7. Permissions

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Glossary of terms

1. **Adulthood** (of shrimp): presence of maturing gonads
2. **Cheating**: a temporary deviation from the normal symbiotic relationship of many different mutualisms, and is represented by a brief exploitation of benefits by one partner of another, with reduced or no reciprocal benefits afforded in return
3. **Cleaner**: the organism doing the cleaning in a cleaning symbiosis
4. **Cleaner punishment**: the reciprocal action of chasing or biting the cleaner by the clinet in response to assumed cheating
5. **Cleaning symbiosis**: a cooperative interspecific behaviour where a cleaner removes and consumes materials that negatively impact a client and is preceded by their communication
6. **Client**: the organism being cleaned in a cleaning symbiosis
7. **Dedicated cleaner**: These cleaners demonstrate a more dedicated cleaning lifestyle than facultative cleaners, and many familiar examples include those species which tend prominent cleaning stations on the reef which are frequented by clients
8. **Epibiont**: all foreseeable organisms that grow on the available space provided by the surface area of another living organism
9. **Facultative cleaner**: not a dedicated cleaner; cleaners with less reliance on cleaning symbiosis as a way of life, but that interact with clients as opportunities present themselves, and quite notably, but not exclusively, as juveniles
10. **Host**: used for parasitological interactions only – the organism being parasitised
11. **Incidental cleaning**: cleaning by one organism of another without precluding communication; not necessarily cooperative
12. **Infestation**: used in this thesis to mean infection by parasites

13. **Jolt, jolting, jolt-rate**: proxy for cheating; the short, sharp reactions of the client to the assumed cheating of the cleaner, often followed by reciprocal cleaner punishment
14. **Life support system**: the collective term for all filtration including biological, mechanical, and ultraviolet light disinfection employed during the experimentation for this thesis. Specifically, these include foam fractionation, biological filtration using bio-balls to increase surface area for bacterial growth, and nitrate export using live algae scrubbers; particle filtration using filter bags; continuous recirculating germicidal ultraviolet light sterilisation.
15. **Obligate**: used for parasitological interactions only – denoting a binding mode of co-existence with a host, without which the parasite will die
16. **Oncomiracidium (idia)**: larval stage of Monogenea
17. **Overdispersed, overdispersion**: the specific statistical term denoting the spread of data, not the ecological term used in parasitology
18. **Symbiosis**: commensalism, mutualism, and parasitism
19. **Theront**: reinfective stage of *Cryptocaryon irritans*
20. **Tomont**: reproductive environmental stage of *Cryptocaryon irritans*
21. **Trophont**: parasitic stage of *Cryptocaryon irritans*